University of Alberta

The Role of the Ileal Lipid-Binding Protein in Bile Acid Transport

by

Eric D. Labonté ©

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Abbreviations

ABCa1	ATP-Binding Cassette Transporter A1
ABCg5	ATP-Binding Cassette Transporter G5
ABCg8	ATP-Binding Cassette Transporter G8
ANOVA	Analysis of Variance
AP2	Adipocyte Lipid-Binding Protein
ASBT	Apical Sodium-Bile Acid Transporter
ATP	Adenosine Triphosphate
BARE	Bile Acid Response Element
BSEP	Bile Salt Export Pump
CA	Cholic Acid
CD	Circular Dichroism
CDCA	Chenodeoxycholic Acid
cDNA	Complimentary Deoxyribonucleic Acid
Chol	Cholesterol
CMV	Cytomegalovirus
CRBP-II	Cellular Retinol-Binding Protein II
CS	Calf Serum
Cyp27	Cholesterol 27α-Hydroxylase
Cyp46a1	Sterol 24-Hydroxylase
Cyp7a1	Cholesterol 7α-Hydroxylase A1
Cyp7b1	Oxysterol 7α-Hydroxylase B1
Cyp8b1	Oxysterol 12α-Hydroxylase B1
DCA	Deoxycholic Acid
dCTP	Deoxycytidine 5'-Triphosphate
DMEM	Dulbecco's Modified Eagle Medium

DNA	Deoxyribonucleic Acid
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
ELSD	Evaporative Light Scattering Detector
FABP	Fatty Acid-Binding Protein
FBS	Fetal Bovine Serum
FPLC	Fast Performance Liquid Chromatography
FXR	Farnesoid X Receptor
FXRE	FXR Response Element
G3PDH	Glyceraldehyde-3-Phosphate Dehydrogenase
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
GC	Gas Chromatography
GCA	Glycocholic Acid
GCDCA	Glycochenodeoxycholic Acid
GDCA	Glycodeoxycholic Acid
GLCA	Glycolithocholic Acid
GUDCA	Glycoursodeoxycholic Acid
HDL	High Density Lipoprotein
HMGCR	3-Hydroxy-3-Methylglutaryl-Coenzyme A Reductase
HPLC	High Performance Liquid Chromatography
I-BABP	Intestinal Bile Acid-Binding Protein
I-FABP	Intestinal Fatty Acid-Binding Protein
ILBP	Ileal Lipid-Binding Protein
IPTG	Isopropylthio-β-D-Galactoside
kDa	Kilodalton
LCA	Lithocholic Acid
LDL	Low Density Lipoprotein
L-FABP	Liver Fatty Acid-Binding Protein
LRH1	Liver Receptor Homologue 1
LXRα	Liver X Receptor α

MALDI-TOF	Matrix Assisted Laser Desorption Ionization-Time of Flight
mILBP	Murine Ileal Lipid-Binding Protein
MRP2	Multidrug Resistant Protein 2
MRP3	Multidrug Resistant Protein 3
MS	Mass Spectrometry
ND	Not Detectable
NMR	Nuclear Magnetic Resonance
nor-CA	Nor-Cholic Acid
nor-DCA	Nor-Deoxycholic Acid
NR1	Nuclear Receptor 1 Family
NS	Not Significant
NTCP	Sodium-Taurocholate Cotransporting Polypeptide
OATP	Organic Anion Transporting Polypeptides
PAGE	Polyacrylamide Gel Electrophoresis
PBS	Phosphate Buffered Saline
PC	Phosphatidylcholine
PCR	Polymerase Chain Reaction
PLTP	Phospholipid Transfer Protein
Q-PCR	Quantitative Polymerase Chain Reaction
RNA	Ribonucleic Acid
rRNA	Ribosomal Ribonucleic Acid
RXR	Retinoic Acid X Receptor
SDS	Sodium Dodecyl Sulphate
SHP	Short Heterodimerization Partner
SREBP1C	Sterol Regulatory Binding Protein 1C
SSC	Sodium Chloride Sodium Citrate
tASBT	Truncated Apical Sodium-Bile Acid Transporter
TCA	Taurocholic Acid
TCDCA	Taurochenodeoxycholic Acid

TCDCA	Taurodeoxycholic Acid
TG	Triglyceride
TLC	Thin-Layer Chromatography
TLCA	Taurolithocholic Acid
TPBS	Tween Phosphate Buffered Saline
TUDCA	Tauroursodeoxycholic Acid
UDCA	Ursodeoxycholic Acid
UV	Ultraviolet
VLDL	Very Low Density Lipoprotein
β-MCA	β-Muricholic Acid
β-TMCA	Tauro- β-Muricholic Acid

Chapter 1

Introduction

1.1 Bile

Bile serves the dual purpose of ridding the body of potentially noxious products and of providing the biological detergents necessary for fat solubilization and digestion. It is produced by the liver and stored in the gallbladder until it can be secreted into the lumen of the intestine upon ingestion of a meal. The human liver secretes over 500 ml of bile each day (Shaffer, 2002). It is a complex mixture consisting of mostly water and containing three lipid groups: bile acids, phospholipids, and cholesterol. Bile acids are the most abundant organic solutes in bile and are the emulsifying agents that facilitate intestinal absorption of dietary lipids and lipid-soluble vitamins. These molecules are themselves efficiently absorbed in the distal portion of the small intestine and can be reused many times before excretion. As a result, despite the fact that the human liver secretes 30 g of bile acids a day, the efficient intestinal reabsorption and the hepatic reuse of these molecules only requires that 0.5 g bile acids per day be newly synthesized from cholesterol. This production of bile acids accounts for about 90% of the cholesterol that is actively metabolized and represents the major path of cholesterol clearance in the body. The recycling of bile acids ensures that bile acid molecules may be used by the body more than once and economizes the metabolic energy investment. However, once cholesterol is converted to a bile acid in the liver, it is dedicated to eventual elimination from the body.

1.2 Dietary Detergents

Bile acids are amphipathic molecules that are derived from cholesterol. In Figure 1-1, the structure of two bile acid species are shown. They are cholic acid (CA) and taurocholic acid (TCA), CA conjugated by taurine at the carboxylic acid moiety. Bile acids differ from their cholesterol

precursors in four important ways (Hofmann, 1999a; Hofmann, 1999b). The first is that the 5-6 double bond of cholesterol is saturated, changing the confirmation of the sterol moiety. The second is that the 3β-hydroxyl group is epimerized to a 3α confirmation. Thirdly, depending on the bile acid species, the sterol ring of a bile acid can be hydroxylated up to three times. Hydroxylation always occurs at the 3 position and may occur either once or twice more at the 6, 7, or 12 positions. The last difference is that the 24-25 bond is oxidatively cleaved in order to give a carboxylic acid moiety, increasing the degree of amphipathicity of this class of lipids. Bile acids are normally conjugated with glycine or taurine, a process that makes them impermeable to cell membranes and permits high concentrations to persist in bile and in the intestinal contents. Furthermore, conjugated bile acids have a much lower pK_a, ensuring that these molecules maintain their negative charge in the pH range present in the small intestine. As such, unconjugated bile acids are physiologically rare since bile acids are converted to a conjugated form immediately after biosynthesis. The hydroxylation of naturally occurring bile acids adds to the amphipathic nature of these molecules, particularly having the hydroxyl groups project on only one side of the sterol ring structure. Therefore, the acid portion of the conjugated amino acid and the facial amphipathic nature of the molecule combine to produce an effective detergent that can solubilize dietary lipids. The plethora of different bile acids in any mammal species is further expanded by the actions of anaerobic bacteria in the gut, which convert the intestinal bile acids into dozens of other bile acid species (Hylemon and Harder, 1998). The physicochemical diversity in bile acid speciation allows for the emulsification of dietary lipids that can vary in hydrophobicity. Table 1-1 summarizes the most important bile acid species including the major bile acids found in humans and mice.

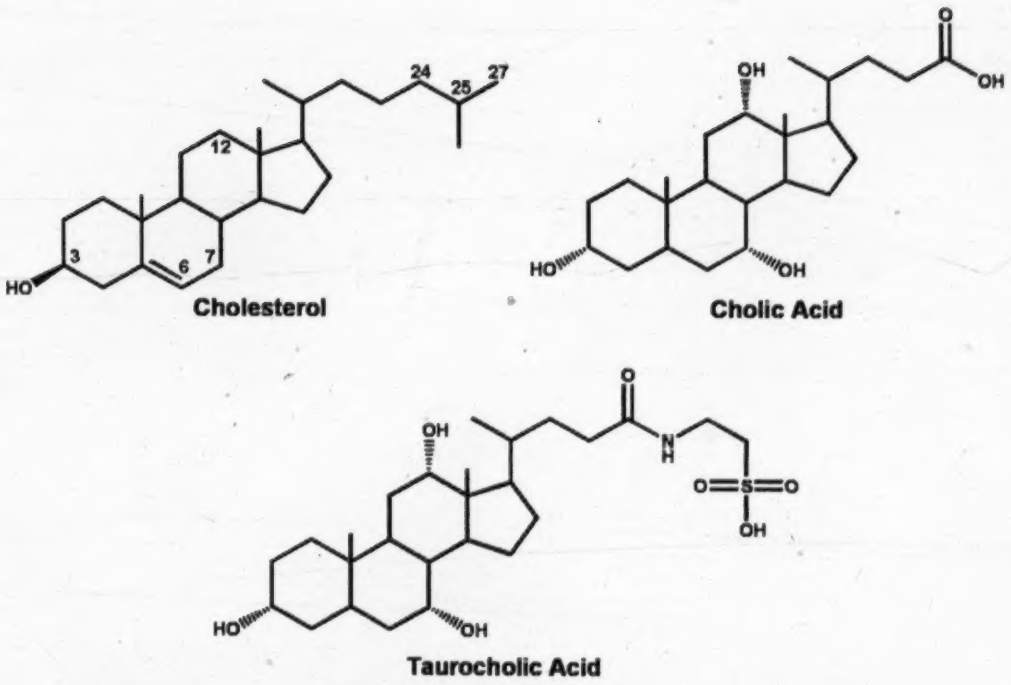

Figure 1-1. The structure of cholesterol, cholic acid, and taurocholic acid. Chemical structures of cholesterol and of two bile acids: cholic acid and taurocholic acid, cholic acid conjugated by taurine. The cholesterol structure shows the convention of numbered carbons of a sterol ring. Cholic acid is hydroxylated at the 3, 7, and 12 positions each in the α orientation.

Table 1-1. Common Physiological Bile acids

Name	Hydroxyl Groups	Conjugated AA
Unconjugated Bile Acids		
Cholic Acid (CA)	3α, 7α, 12α	-
Chenodeoxycholic Acid (CDCA)	3α, 7α	-
β-Muricholic Acid (β-MCA)	3α, 6β, 7β	-
Deoxycholic Acid (DCA)	3α, 12α	-
Ursodeoxycholic Acid (UDCA)	3α, 7β	-
Lithocholic Acid (LCA)	3α	-
Major Human Bile Acids		
(30-40%) Glycocholic Acid (GCA)	3α, 7α, 12α	Glycine
(30-40%) Glycochenodeoxycholic Acid (GCDCA)	3α, 7α	Glycine
(20-30%) Glycodeoxycholic Acid (GDCA)	3α, 12α	Glycine
Major Murine Bile Acids		
(40-50%) Taurocholic Acid (TCA)	3α, 7α, 12α	Taurine
(40-50%) Tauro-β-muricholic Acid (TMCA)	3α, 6β, 7β	Taurine
Other Physiological Bile Acids		
Taurochenodeoxycholic Acid (TCDCA)	3α, 7α	Taurine
Taurodeoxycholic Acid (TDCA)	3α, 12α	Taurine
Tauroursodeoxycholoic Acid (TUDCA)	3α, 7β	Taurine
Taurolithocholic Acid (TLCA)	3α	Taurine
Glycoursodeoxycholic Acid (GUDCA)	3α, 7β	Glycine
Glycolithocholic Acid (GLCA)	3α	Glycine

Trivial names of bile acids and their common abbreviations. Approximate percentage of total bile acids found in humans and mice are shown in parenthesis. The position and orientation of each hydroxyl group is shown as well as the conjugated amino acid (aa) moiety.

Two major physiological functions of bile acids are now well established. The first is that, in mammals, bile acids represent the major pathway of cholesterol elimination from the body (Dietschy, 1997). Cholesterol is converted to bile acids, which promotes the biliary secretion of free cholesterol, ultimately leading to fecal riddance of both neutral and acidic sterols. Inborn defects in the conversion of cholesterol to bile acids can

cause severe hepatic or systemic disease. The manipulation of this process has long been investigated as a means to reduce plasma cholesterol. The second major physiological contribution of bile acids is the enhancement of the emulsification and absorption of lipids. Bile acids aid in the dispersement of large dietary lipid globules by forming mixed micelle complexes. The markedly increased surface areas of these micelles are imbedded with bile acids so that the polar face and acid group are facing outward, effectively allowing soluble access to a hydrophobic milieu. The emulsion of lipids renders them more accessible to secreted pancreatic lipases and facilitates their absorption by luminal enterocytes of the small intestine. Cholestasis, the lack of bile flow to the small intestine, all but abrogates lipid absorption. Bile acids may also affect gastrointestinal motility and mucin secretion (Hofmann, 1999b).

1.3 Bile Acid Biosynthesis

Bile acids are the water-soluble end products of cholesterol metabolism. Cholesterol is converted into bile acids by pathways that involve the concerted effort of 17 different enzymes, many of which are preferentially expressed in the liver (Russell and Setchell, 1992; Schwarz *et al.*, 1998a; Bjorkhem and Eggertsen, 2001; Russell, 2003). As such, some bile acid biosynthesis may be initiated in other tissues by the hydroxylation of cholesterol but largely occurs and is controlled by the liver. Bile acid synthesis involves enzymes expressed in four cellular compartments requiring shuttling for intermediates. The steps leading to synthesis of bile acids are summarized in Figure 1-2 and Figure 1-3: (*a*) the initiation of synthesis by the hydroxylation of cholesterol, usually at the 7α position; (*b*) further modifications to the ring structures including saturation, epimerization, and possible further hydroxylation; (*c*) oxidation of the side chain; and (*d*)

conjugation of the bile acid by hepatic bile acid CoA:amino acid *N*-acyltranferase with either a glycine or taurine amino acid (Falany *et al.*, 1994). The exact order of steps in the biosynthetic pathway remains unclear because many of the intermediates serve as substrates for more than one biosynthetic enzyme. Nevertheless, two separate cholesterol hydroxylation initiation pathways have been identified: the classical and the alternate.

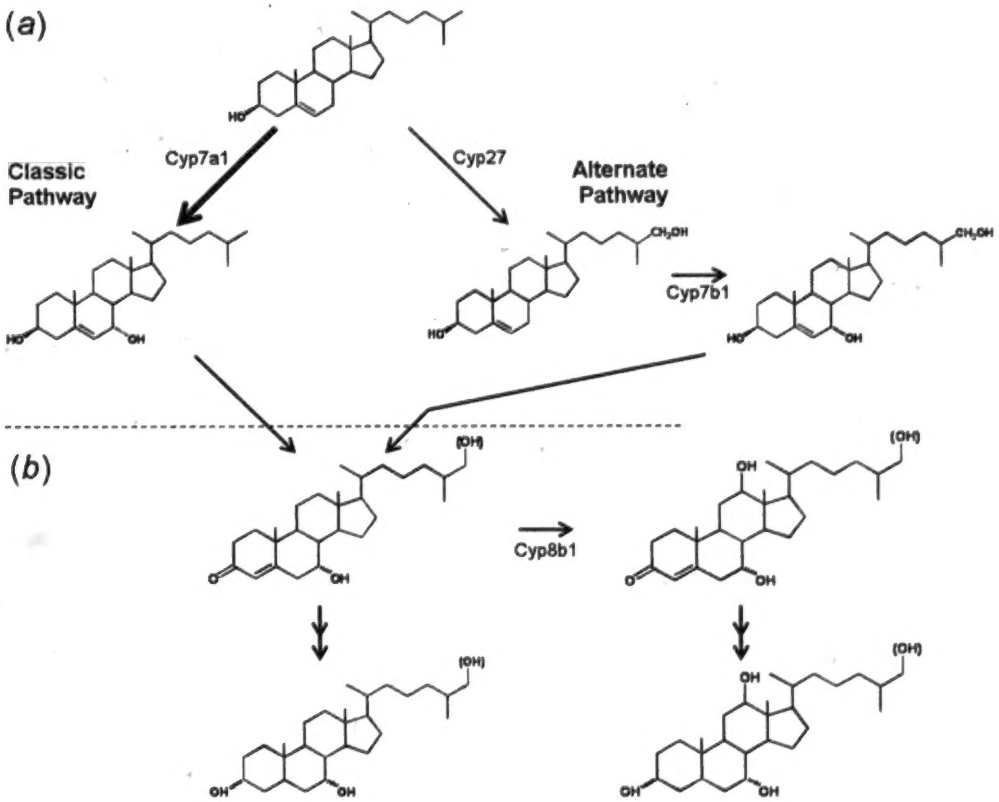

Figure 1-2. Bile acid biosynthesis: hydroxylation and sterol ring modifications. The initial hydroxylation step (a) involves either the classic pathway or the alternate pathway. The classic pathway represents the bulk of bile acid production and is initiated by cholesterol 7α-hydroxylase (Cyp7a1). The alternate pathway is usually initiated by cholesterol 27-hydroxylase (Cyp27) but can also start with a sterol 24- or 25-hydroxylase. The alternate pathway achieves hydroxylation at the 7 position via the oxysterol 7α-hydroxylase (Cyp7b1). The ring modification step (b) uses substrate from either the classic or alternate pathways and is unaffected by substrate hydroxylation at the 24, 25, or 27 positions. Epimerization of the hydroxyl group at the 3 position and saturation of the sterol ring is achieved. Some intermediates are acted on by sterol 12α-hydroxylase (Cyp8b1) adding to the species diversity of bile acids.

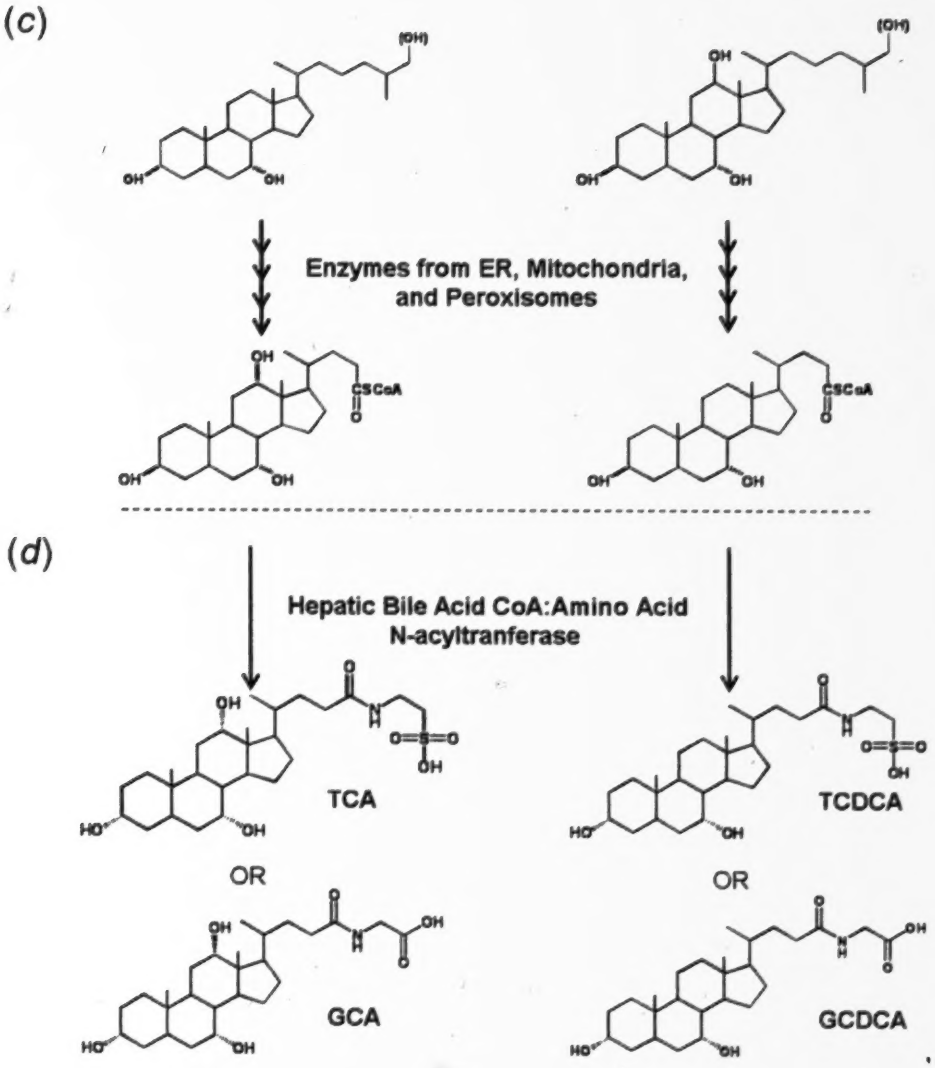

Figure 1-3. Bile acid biosynthesis: Oxidative side chain cleavage and amino acid conjugation. The side chain cleavage step (c) involves 6 enzymes expressed in the endoplasmic reticulum (ER), mitochondria, and peroxisomes. Intracellular shuttling is required by biosynthetic enzymes found in the cytosol. Conjugation of glycine or taurine (d) is achieved via the hepatic bile acid CoA:Amino Acid *N*-acyltransferase expressed in peroxisomes. The result is four species of bile acids: taurocholic acid (TCA), taurochenodeoxycholic acid (TCDCA), glycocholic acid (GCA), and glycochenodeoxycholic acid (GCA).

1.3.1 The Classical Bile Acid Biosynthetic Pathway

The first and rate limiting enzyme of the classical pathway is the cytochrome P450 enzyme cholesterol 7α-hydroxylase (Cyp7a1). It is a microsomal enzyme that is expressed exclusively in the liver. P450 enzymes are mixed function mono-oxidases located in the microsomes and mitochondria of hepatocytes. Six of the 17 enzymes involved in bile acid biosynthesis are members of the P450 superfamily. Cyp7a1 has a low turnover number and prefers cholesterol as substrate. *Cyp7a1* null mice have a dramatic postnatal deficiency in bile acid production that disrupts bile acid function in the gut (Schwarz *et al.,* 1996). Furthermore, Cyp7a1-deficient mice display a drastic decrease in post-natal survival that is remedied by the inclusion of CA in the chow (Ishibashi *et al.,* 1996). This observation has led to the conclusion that despite the presence of the alternate bile acid biosynthetic pathway, the classical pathway and Cyp7a1 have a central role in the regulation of overall bile acid production (Russell and Setchell, 1992). Cyp7a1 is regulated by many factors; however, transcriptional regulation from feed-forward activation or feed-back inhibition is an important tightly regulated mechanism for bile acid biosynthesis. Dietary and endogenous sources of cholesterol or oxysterols have long been observed to increase both Cyp7a1 activity and mRNA abundance (Li *et al.,* 1990; Dueland *et al.,* 1993). This is similar to 3-hydroxy-3methylglutaryl CoA reductase (HMGCR), considered the rate limiting step for cholesterol biosynthesis, where studies have shown increased dietary cholesterol reduces both the activity and mRNA levels of HMGCR (Ness and Chambers, 2000). The activation of *Cyp7a1* transcription by cholesterol involves the liver X receptor α (LXRα), an oxysterol-activated nuclear receptor (Janowski *et al.,* 1996; Lehmann *et al.,* 1997). Similarly, administration of bile acid-binding resin has been shown to decrease *Cyp7a1* mRNA abundance (Li *et al.,* 1990; Dueland *et al.,* 1993). Further studies of Cyp7a1 expression

elucidated that dietary regulation of the corresponding gene in rodents was primarily transcriptional (Hoekman *et al.*, 1993; Stravitz *et al.*, 1993). More recently, it has been determined that *Cyp7a1* mRNA abundance is regulated by an indirect mechanism. Through the activation of the farnesoid X receptor (FXR) nuclear receptor by bile acids (Makishima *et al.*, 1999; Wang *et al.*, 1999), increased short heterodimerization partner (SHP) gene expression has been shown to inhibit the transcriptional activation of *Cyp7a1* (Goodwin *et al.*, 2000; Lu *et al.*, 2000). Since then, several genes important to bile acid metabolism have been shown to be transcriptionally inhibited by this mechanism.

1.3.2 The Alternate Bile Acid Biosynthetic Pathway

The alternate pathway utilizes oxysterols produced in the liver and from peripheral tissues. Oxysterols are carried to the liver via the circulation where conversion to bile acids is completed. Hydroxylation of cholesterol at three different positions on the side chain produces 24-hydroxycholesterol, 25-hydroxyxholesterol, and 27-hydroxycholesterol. Although sterol 24-hydroxylase (Cyp46a1) is expressed in neurons and is important to cholesterol metabolism in the brain (Bjorkhem *et al.*, 1999; Lund *et al.*, 1999), the overall contribution of this hydroxylase to bile acid biosynthesis is minimal (Bjorkhem *et al.*, 2001). The microsomal sterol 25-hydroxylase is not a cytochrome P450 enzyme but an evolutionarily older enzyme and utilizes oxygen and a diiron-oxygen cofactor to hydroxylate that is ubiquitously expressed (Lund *et al.*, 1998). The contribution of sterol 25-hydroxylase to the bile acid biosynthetic pathway is unknown but thought to be limited. Since the most abundant oxysterol in plasma of both mice (Li-Hawkins *et al.*, 2000) and humans (Dzeletovic *et al.*, 1995) is 27-hydroxycholesterol, sterol 27-hydroxylase (Cyp27) is thought to be the most important hydroxylase for

the alternate pathway. Furthermore, this enzyme is also known to hydroxylate at the 24 and 25 positions of a sterol (Lund *et al.*, 1993). It is estimated that the alternate pathway via Cyp27 hydroxylation contributes 25% of the total bile acids utilized in a mouse and the classical pathway produces 75% (Schwarz *et al.*, 1998b; Schwarz *et al.*, 2001). In order to continue bile acid biosynthesis, hydroxylation at the 7 position is required. Therefore, oxysterol 7α-hydroxylase (Cyp7b1) is required to allow oxysterols from the alternate pathway to be processed. Products of the alternate pathway differ from those of the classical pathway in that they are hydroxylated at the 24, 25, or 27 positions. Cyp27 activity is important for side chain cleavage (*c*) in later steps and therefore premature hydroxylation from the alternate pathway does not interfere with bile acid biosynthesis.

1.3.3 Bile Acid Speciation

Two basic bile acids are produced by most vertebrate species before conjugation. One is usually cholic acid (CA) and the other is chenodeoxycholic acid (CDCA) for rats, humans, and hamsters; β-muricholic acid (β-MCA) for mice; ursodeoxycholic acid (UDCA) for bears; or hyodeoxycholic acid for pigs. The level of sterol 12α-hydroxylase (Cyp8b1) activity determines the relative ratio of the two. Cyp8b1 hydroxylates at the 12 position and is responsible for the production of CA. Loss of Cyp8b1 in mice eliminates CA from the bile acid pool and increases the level of β-MCA (Li-Hawkins *et al.*, 2002). The method of β-MCA synthesis remains unknown but murine Cyp8b1 is thought to play a role. In humans, deoxycholic acid (DCA) and lithocholic acid (LCA) are derived from the removal of hydroxyl groups at position 7 or 12 by intestinal bacteria. Bile acid recycling accounts for the relatively high levels (20-30%) of DCA in the human bile acid pool

(Table 1-1). Conjugation also adds to the pluralization of bile acids, although glycine is preferred in humans while taurine is preferred in mice

1.4 The Enterohepatic Circulation of Bile Acids

Bile acid homeostasis involves hepatic biosynthesis and vectorial transport in several organs. After assisting lipid digestion, bile acids are absorbed mostly in the enterocyte of the ileum and returned to the liver via portal circulation for reuse. This cycling of bile acids from the liver to the intestine and back is called the enterohepatic circulation (Figure 1-4) and permits each bile acid molecule to be used many times depending on the animal species (Hofmann, 1999a, b). Regulation of the enterohepatic circulation can occur either at the level of hepatic biosynthesis or controlled absorption at the ileum. Thus, the size of the bile acid pool can be regulated by controlling either the synthesis (Vlahcevic *et al.*, 1999; Xu *et al.*, 1999) or the excretion of bile acids (Xu *et al.*, 2000; Shneider, 2001). The strategies used to regulate the enterohepatic circulation can vary between species. The enterohepatic cycling of bile acids requires the concerted efforts of a number of proteins to transport bile acids. The transporters most commonly associated with bile acid transport are described in this section and shown in Figure 1-5.

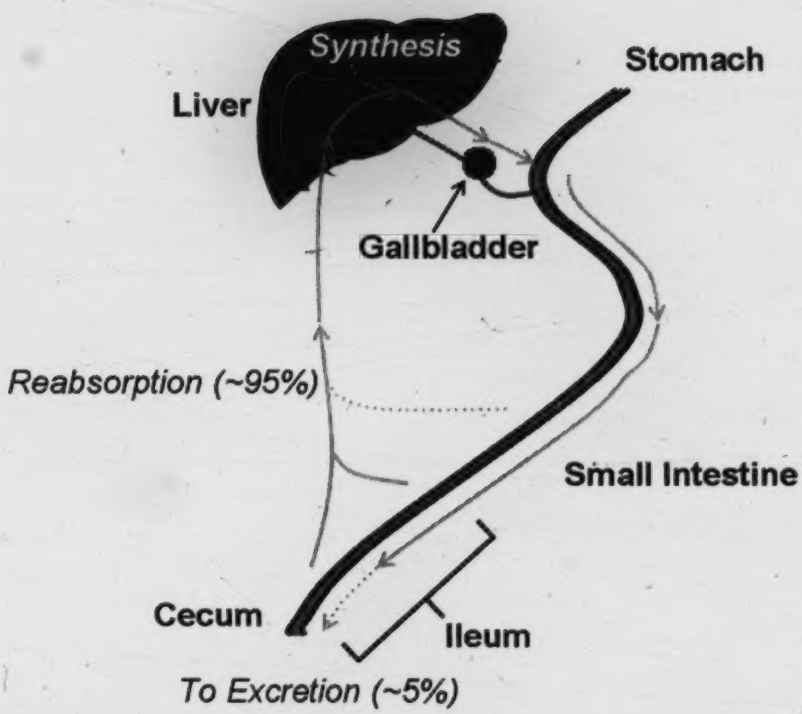

Figure 1-4. The enterohepatic cycling of bile acids. The arrows follow the path of bile acids in the enterohepatic circulation of a human. When reabsorbed at the ileum, only approximately 5% is left to excretion. Adapted from (Dawson and Oelkers, 1995).

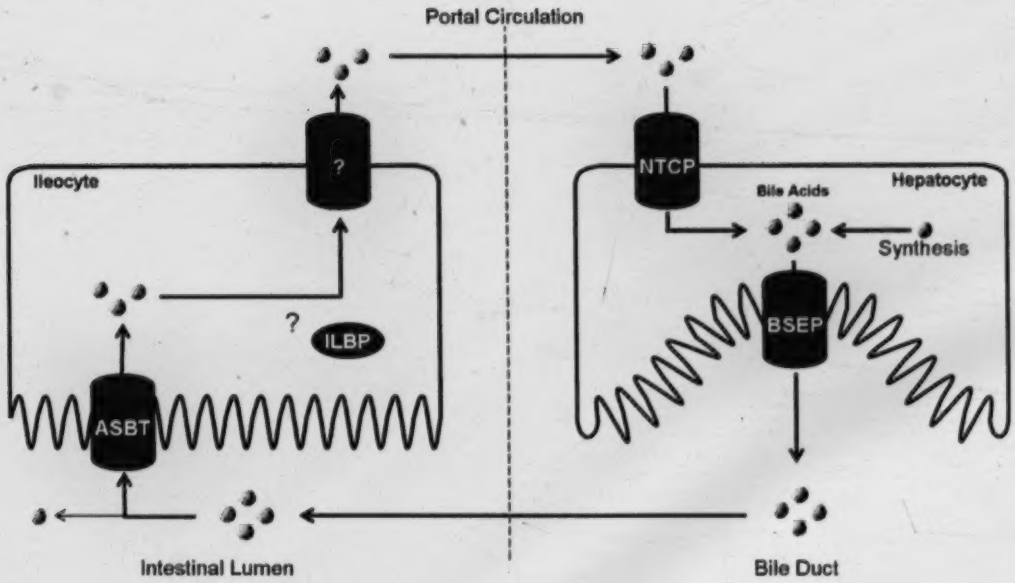

Figure 1-5. Major bile acid transporters in the enterohepatic circulation. Bile acids are secreted into the canalicular space between hepatocytes by the bile salt export pump (BSEP) as a component of bile. When bile is secreted into the intestinal lumen from the common bile duct, efficient bile acid recovery is mediated via the apical sodium-dependent bile acid transporter (ASBT). The ileal lipid-binding protein (ILBP) is thought to be involved in the intracellular trafficking of bile acids in the ileocyte. Bile acids are then secreted into portal circulation via unknown or disputed transporter(s). Bile acids are then recovered from portal circulation by the liver mostly by the sodium-dependent taurocholate cotransporting polypeptide (NTCP). Synthesis of bile acids makes up for any loss and the cycle begins anew. Arrows indicate the direction of bile acid transport within the enterohepatic circulation or elimination from the body.

1.4.1 Bile Secretion

As mentioned above, bile is predominantly composed of bile acids, phospholipids and cholesterol. Hepatic bile acid secretion, which drives bile formation (Nathanson and Boyer, 1991), is mediated by an ATP-binding

cassette (ABC) transporter called the bile acid export pump (BSEP) found in the canalicular membranes of hepatocytes (Childs *et al.*, 1995; Gerloff *et al.*, 1998). Hepatocytes do not accumulate potentially cytotoxic bile acids. Immediately after the uptake from portal blood of gut-absorbed bile acids, they are efficiently shuttled from the basolateral to the apical or canalicular membrane for bile secretion. Under normal physiological conditions, the bile acid concentration in hepatocytes is considered to be relatively low (<1 μM) (Setchell *et al.*, 1997; Hofmann, 1999b). In mammalian species, the canalicular bile acid concentration is about 1000-fold higher than that in portal blood, emphasizing the need for active vectorial transport at the apical membrane. Mutations in the human *BSEP* gene, a member of the multidrug resistance protein (MRP) family, are associated with subtype II familial progressive intrahepatic cholestasis (Strautnieks *et al.*, 1998). As such, the 160 kDa BSEP is considered the major bile acid export protein in hepatocytes (Kullak-Ublick *et al.*, 2004). Two other members of the MRP family, MRP2 and MRP3, transport sulfated and glucuronidated bile acids and are thought to play a limited role in overall hepatic bile acid secretion.

Secreted bile acids promote the extraction of hepatic phospholipids, mainly phosphatidylcholine (Meier and Stieger, 2000), and cholesterol. Together, bile acids and phosphatidylcholine allow for the solubilization of cholesterol which takes the form of mixed unilamellar vesicles and micelles. This solubilization of cholesterol through micelle formation enables further cholesterol efflux from the apical hepatocyte membranes during bile formation. Mixed micelle formation also protects the biliary epithelia from the toxic effects of bile acids (Kuipers *et al.*, 1997). Recently, a heterodimeric ABC transporter that is encoded by two oppositely oriented genes, termed *ABCg5* and *ABCg8*, has been identified as an apical transporter involved in cholesterol excretion into bile (Yu *et al.*, 2002a, b).

As bile flow is conveyed by the biliary tract, the biliary epithelium actively modifies the volume and composition of canalicular bile, mainly

through vectorial transport of water, electrolytes, and macromolecules. Bile passing through the biliary tree is concentrated for bile acids. In animals with a gallbladder, bile may be stored interdigestively, resulting in intermittent intestinal bile flow and in further bile acid concentration. Typically, bile acids in bile taken from gallbladders may be 5- to 10-fold more concentrated than bile acids from hepatic biliary secretion. Upon ingestion of a meal, the gallbladder contracts and releases bile into the duodenum in response to signaling from the hormone cholecystokinin (Hofmann, 1999b). By contrast, animals with no gallbladder, such as rats, exhibit continuous intestinal bile flow but also maintain the ability to concentrate bile acids in the bilary tree and common bile duct.

1.4.2 Intestinal Bile Acid Reclamation

Intestinal uptake of bile acids can occur by both passive and active mechanisms (Aldini *et al.,* 1996). The result is that greater than 95% of secreted bile acids are recovered in humans. This is a surprising recovery effciency considering that it is widely accepted that humans, and most mice strains, absorb only 30% to 70% of dietary cholesterol (Dietschy and Turley, 2002). The process and efficiency of bile acid reclamation depends largely on the hydroxylation and conjugation states of a particular bile acid. Unconjugated and protonated bile acids may be reclaimed along the whole axis of the small intestine by means of passive diffusion. However, conjugated bile acids are unable to pass through cellular membranes and require active transport to gain entry to a cell (Hofmann, 1999b). While there is evidence of a jejunal anion exchange transport of bile acid (Lewis and Root, 1990; Amelsberg *et al.,* 1999; Walters *et al.,* 2000), the majority of bile acid reclamation occurs in the terminal ileum (Buchwald *et al.,* 1990; Amelsberg *et al.,* 1996; Ishibashi *et al.,* 1996). For example, re-sectioning of

¼ or more of the human ileum results in both bile acid and fatty acid malabsorption with severe diarrhea and steatorrhea (Hofmann and Poley, 1972). Bile acid absorption occurring predominantly in the ileum was first observed by Tappeiner in 1878 (Tappeiner, 1955) and has only been confirmed relatively recently.

Active transport in the ileum has been revealed by kinetic analysis to be a sodium-dependent carrier-mediated process (Wong *et al.*, 1994). Using an expression cloning approach based on this finding, the apical sodium-dependent bile acid transporter (ASBT) cDNA was cloned from hamsters (Shneider *et al.*, 1995). ASBT is expressed on the apical membranes of the ileal section of the small intestine, in cholangiocytes of the liver (Alpini *et al.*, 1997), and in renal proximal convoluted tubule cells (Christie *et al.*, 1996). The cDNAs for this 348 amino acid transporter have been cloned from human, rat, mouse, and rabbit (Hagenbuch *et al.*, 1991; Christie *et al.*, 1996; Alpini *et al.*, 1997; Craddock *et al.*, 1998). Cultured cells expressing the ASBT cDNA initiate sodium-dependent conjugated and unconjugated bile acid uptake (Craddock *et al.*, 1998). Further, mutations in the human *ASBT* gene have been observed to cause bile acid malabsorption (Oelkers *et al.*, 1997) and the ontogenic expression of ASBT in rats has been observed to coincide with the onset of active ileal bile acid uptake (Shneider *et al.*, 1995). ASBT inhibitors have been seen to mimic the effect of feeding bile acid binding resin and reducing bile acid recovery in the intestine (Lewis *et al.*, 1995; Root *et al.*, 1995). More recently, studies utilizing the ASBT-deficient mice demonstrated that ASBT is essential for efficient intestinal absorption of bile acids and that alternative absorptive mechanisms are unable to compensate for the loss of ASBT (Dawson *et al.*, 2003). These observations suggest that ASBT is the primary transporter that mediates sodium-dependent bile acid transport. ASBT has been found to preferentially bind conjugated bile acids (Kramer *et al.*, 1999), which is consistent with known ileal bile acid uptake kinetics.

Studies involving the brush border membranes of the ileum have elucidated much about bile acid absorption in the gut. Using photoactive bile acid probes, a membrane integrated 93 kDa and a cytosolic 14 kDa protein were identified in active transport of bile acids in studies of rat and rabbit ileal cells (Lin *et al.,* 1990; Kramer *et al.,* 1993). The larger protein was later recognized as two covalently bonded ASBT molecules (Kramer *et al.,* 1997), while the smaller protein was later identified as the previously characterized ileal lipid-binding protein (ILBP) (Gantz *et al.,* 1989; Sacchettini *et al.,* 1990; Gong *et al.,* 1994). Using radiation-inactivation analysis, it was further determined that ILBP is intimately associated with ASBT in large homotetrameric complexes composed of four ASBT/ILBP pairs (Kramer *et al.,* 1995; Kramer *et al.,* 1997). This same group later confirmed an interaction between ASBT and ILBP by using photolabile-dimeric bile acid transport inhibitors (photoblockers) (Kramer *et al.,* 1997). Furthermore, the binding of bile acids to this ileal transport complex seemed to improve the affinity of ILBP for bile acids (Kramer *et al.,* 1998). This would suggest that ILBP could be charged with bile acids with the assistance of the ileal transport complex and ASBT for the purposes of intracellular trafficking. However, since ASBT transports bile acids in tissue culture cell models in the absence of ILBP (Wong *et al.,* 1994; Craddock *et al.,* 1998) and ASBT functions in cholangiocytes despite the lack of ILBP expression (Christie *et al.,* 1996; Craddock *et al.,* 1998), ILBP is not a requirement for ASBT activity. Furthermore, ontogenic expression of ILBP is not detected in rats until post-natal day 17 (Gong *et al.,* 1996), well after gut bile acid reclamation has commenced. However, ILBP interacting with ASBT at the cytoplasmic face of the enterocyte suggests a direct function in active ileal bile acid absorption. The structure, regulation, and function of ILBP, ASBT, and FXR in ileal bile acid transport are further discussed in section 1.5.1.

1.4.3 Bile Acid Efflux from Ileocytes

Basolateral secretion of bile acids in enterocytes also remains to be characterized. Earlier photoaffinity labeling studies indicated that bile acid movement across intestinal basolateral membranes involved an anion exchanger (Weinberg *et al.*, 1986; Lin *et al.*, 1988). Subsequent studies indicated the presence of membrane integral proteins that bound bile acids (Lin *et al.*, 1988; Simon *et al.*, 1990). More recently, two proteins have been identified as possible intestinal basolateral transporters of bile acids. The first is an ABC transporter in the MRP subfamily, MRP3 (ABCC3), which is known to transport bile acids in other tissues. MRP3 has been found to be expressed in the basolateral membrane of enterocytes, to efflux bile acids, and to be upregulated in the presence of bile acids (Kool *et al.*, 1997; Hirohashi *et al.*, 1998; Konig *et al.*, 1999; Inokuchi *et al.*, 2001). However, human MRP3 has a lower affinity for conjugated bile acids than rat MRP3, suggesting that the overall contribution of human MRP3 to basolateral bile acid efflux might be minimal (Akita *et al.*, 2002; Zelcer *et al.*, 2003). The second protein identified as a putative bile acid transporter is a truncated ASBT (tASBT) (Lazaridis *et al.*, 2000). This product of alternative splicing has been shown to have bile acid secretion activity in Xenopus oocytes expressing tASBT and is expressed in the basolateral membrane of enterocytes. The tASBT is a 19 kDa protein expressed in cholangiocytes, ileum, and kidney. However, the physiological importance of this transporter is yet to be determined.

1.4.4 Hepatic Bile Acid Uptake

The liver efficiently extracts bile acids from the portal circulation, where they are mostly bound to lipoproteins, such as high density lipoprotein

(HDL) and albumin. More than 80% of conjugated bile acids undergo single-pass extraction by the liver. The uptake efficiency is aided by the fact that hepatocyte membranes are approximately 85% basolateral and 15% canalicular. Given that human canalicular spaces have bile acid concentrations of 20-50 mM and the bile acid concentration of the portal circulation is 20-50 μM, hepatocytes must constantly transport bile acids from blood against a steep concentration gradient. The basolateral membrane of a hepatocyte has both sodium-dependent and –independent bile acid transport systems. However, hepatic uptake of conjugated bile acids is mediated predominantly via a sodium co-transport mechanism (Anwer and Hegner, 1978; Meier, 1995). The sodium-taurocholate co-transporting polypeptide (NTCP), a membrane integrated serine/threonine phosphorylated glycoprotein (Mukhopadhyay *et al.,* 1998) with seven transmembrane domains (Hagenbuch *et al.,* 1991; Ananthanarayanan *et al.,* 1994; Stieger *et al.,* 1994; Hagenbuch, 1997; Love and Dawson, 1998), is the primary sodium-dependent transporter of the hepatocyte basolateral surface (Nathanson and Boyer, 1991; Anwer, 1993). NTCP belongs to the same transporter family as ASBT, and the human protein consists of 349 amino acids with a high affinity for TCA (Hagenbuch and Meier, 1994). Both ASBT and NTCP prefer conjugated bile acids as substrate (Schroeder *et al.,* 1998; Agellon and Torchia, 2000) and are similar in structure (Shneider, 2001). However, NTCP has a broad substrate specificity compared to ASBT, which facilitates the clearance of a wide variety of organic compounds from the circulation for disposal into bile (Meier *et al.,* 1997; Kouzuki *et al.,* 1998; Kramer *et al.,* 1999). The overall contribution to hepatic bile acid recovery of other sodium-dependent transporters is considered small (Hagenbuch *et al.,* 1996; Kouzuki *et al.,* 1998).

In addition to the sodium-dependent bile acid uptake mediated largely by NTCP, sodium-independent hepatic absorption of bile acids is mediated by the organic anion transporting polypeptides (OATP) (Tirona and Kim,

2002; Hagenbuch and Meier, 2003). Sodium-independent bile acid transport can account for most of the uptake of intestinal bacteria-derived unconjugated bile acids in the liver (Hagenbuch and Meier, 1996). Members of the OATP superfamily transport a wide variety of organic anions including bile acids. Both rodent and human hepatocytes express three members of the OATP superfamily at appreciable levels. However, the expression of OATP family members is not restricted to the liver, reinforcing the importance of OATPs in the cellular transport of non-bile acid molecules. The relative physiological importance of OATPs to hepatic uptake of bile acids remains unknown.

1.5 Bile Acid Transport in Ileocytes

Despite the ileum being important to bile acid conservation, very few proteins expressed in the ileocyte are known to interact with bile acids. Although the apical uptake and ASBT activity are relatively well characterized, the binding, intracellular shuttling, and basolateral transport of bile acids are less well known. ILBP function has yet to be determined and FXR target genes in the ileocyte are only recently becoming apparent. In this section, the elements of bile acid transport, binding, and gene regulation are examined via ASBT, ILBP, and FXR, respectively.

1.5.1 The Apical Na^+-Bile Acid Transporter (ASBT)

Structure – ASBT is an N-glycosylated protein with an apparent molecular weight of 46 kDa and consists of 348 amino acids in human, mouse, and rat (Shneider *et al.*, 1995; Wong *et al.*, 1995). Although the mass of the protein calculated from the amino acid sequence is 38 kDa, the addition of carbohydrate chains and the putative homodimerization via

disulfide bonds often causes this transporter to have an aparent molecular weight of 99 kDa, despite the reducing conditions involved in SDS-PAGE. Stronger reducing conditions than those described in standard SDS-PAGE protocols are required to view this protein at its correct molecular weight. Although originally thought to be a 7 transmembrane domain protein, a recent study suggested that ASBT has nine transmembrane domains with an extracellular N-terminal segment (Hallen *et al.*, 1999; Kramer *et al.*, 2001; Hallen *et al.*, 2002). The C-terminal segment has been identified as the binding site for bile acids and localized to the terminal 56-67 amino acids (Kramer *et al.*, 2001).

Function – ASBT is a sodium-bile acid cotransporter that is expressed on the sinusoidal or apical surface of a cell (Dawson and Oelkers, 1995). This transporter has been shown to prefer conjugated bile acids as substrate (Kramer *et al.*, 1999). ASBT is the major transporter of bile acids in the gut and is functionally reviewed in section 1.4.2.

Regulation – The basis of ASBT regulation appears to be both transcriptional and post-transcriptional. Intestinal ASBT expression is limited to the terminal 30% and 15% of the rat and human small intestine, respectively (Coppola *et al.*, 1998; Stelzner *et al.*, 2000). However, specific patterns of adaption of ASBT expression have been observed in various models of intestinal resection (Coppola *et al.*, 1998; Stelzner *et al.*, 2000). In all species tested, intestinal ASBT mRNA and protein is repressed postnatally and upregulated in the ileum during normal development (Shneider *et al.*, 1995). This pattern is reminiscent of the developmental expression of ILBP in the mouse (Crossman *et al.*, 1994). Studies have shown that corticosteroids and thyroid hormone can induce the precocious expression of the bile acid transporter (Barnard and Ghishan, 1986; Heubi, 1986; Shneider *et al.*, 1993). Change in the stability of ASBT mRNA is also

associated with the ontogenic expression of this transporter (Shneider *et al.*, 1995; Christie *et al.*, 1996). In adult rats, *ASBT* gene expression has been shown to be upregulated by corticosteroids and downregulated in animal models by ileal inflammation (Nowicki *et al.*, 1997; Sundaram *et al.*, 1998). Recently, ASBT mRNA expression was also shown to be induced by glucocorticoids in humans (Jung *et al.*, 2004). However, a recent study has shown that hepatocyte nuclear factor-1α is critical for basal expression (Shih *et al.*, 2001). Another study has located two AP-1 sites in the proximal promoter of ASBT that enhance promoter activity when bound by c-jun and c-fos (Chen *et al.*, 2001). Therefore, transcriptional regulation of the bile acid transporter by AP-1 elements may also be significant.

The bile acid responsiveness of the *ASBT* gene is a subject of controversy and is species specific. The few studies in mice (Torchia *et al.*, 1996a) and rabbits (Xu *et al.*, 2000) have shown bile acid negative and positive feedback of bile acid transport, respectively. In a recent study, human ASBT was found to be negatively transcriptionally regulated by bile acids through a FXR-mediated, SHP-dependent effect upon RXR activation of the *ASBT* gene (Neimark *et al.*, 2004). Another recent study has confirmed the negative feedback regulation of ASBT in mice and has also identified a FXR-mediated, SHP-dependent transcription inhibition mechanism similar to Cyp7a1 (Chen *et al.*, 2003). In rats, ASBT has been observed to be upregulated, downregulated, and unchanged by bile acids in many conflicting studies (Lillienau *et al.*, 1993; Higgins *et al.*, 1994; Dumaswala *et al.*, 1996; Arrese *et al.*, 1998; Coppola *et al.*, 1998). However, one of these studies demonstrates that transcriptional expression of ASBT does not always correlate with bile acid uptake activity (Sauer *et al.*, 2000). These data suggest that the non-translational regulationary mechanisms of ASBT may be important. In addition, this study demonstrated that the level of homodimerization of the bile acid transporter does not correlate with total ASBT expression but seems to be a function of intracellular and extracellular

bile acid concentration. This suggests that dimerization of ASBT is not a function of ASBT expression but a form of post-transcriptional regulation by bile acids.

1.5.2 The Ileal Lipid-Binding Protein (ILBP)

Intracellular transport across the enterocyte, from the apical to the basolateral membrane, is poorly understood. In attempting to recognize potential candidates that bind bile acids through photoaffinity labeling, a 14 kDa protein was identified as the major cytosolic bile acid binder (Lin *et al.*, 1990; Lin *et al.*, 1991). This protein was later characterized and termed the intestinal bile acid binding protein (I-BABP) (Gantz *et al.*, 1989; Gong *et al.*, 1994). I-BABP is also known as the ileal lipid-binding protein after it was characterized in mice (Sacchettini *et al.*, 1990). The ILBP/I-BABP protein was later found to bind fatty acids in addition to bile acids (Sacchettini *et al.*, 1990; Miller and Cistola, 1993; Gong *et al.*, 1994; Fujita *et al.*, 1995). Therefore, because of its dual binding characteristics, ILBP is a more accurate term and will be used for the rest of this thesis. ILBP is a member of the intracellular lipid-binding gene family (Bernlohr *et al.*, 1997; Storch and Thumser, 2000) and is expressed in the terminal ileum of the small intestine (Oelkers and Dawson, 1995; Watanabe *et al.*, 1995), which coincides with the expression of ASBT (Agellon and Torchia, 2000). In rats, ILBP is expressed abundantly in the ileum and somewhat less in cholangiocytes, ovaries, and adrenal gland (Iseki *et al.*, 1993; Gong *et al.*, 1994; Sato *et al.*, 1995; Alpini *et al.*, 1997). The purpose of expression in these tissues is also unknown.

Structure - Similar to other members of the intracellular lipid-binding protein family, ILBP is composed of a water-filled, clam-like structure and two

parallel α-helices forming a lid (Lucke *et al.*, 1996; Lucke *et al.*, 1999; Lucke *et al.*, 2000; Kramer *et al.*, 2001). The tertiary shape of the clam was revealed by NMR spectroscopic studies to be two orthogonal β-sheets consisting of ten anti-parallel β-strands. ILBP was determined to be relatively more flexible and contain a weaker hydrogen-bonding network in comparison to other homologous members of the intracellular lipid-binding family (Lucke *et al.*, 1996; Lucke *et al.*, 1999). The relative flexibility of ILBP may explain its broader substrate specificity. Further, since bile acids are more rigid molecules in comparison to other lipids, flexibility of the protein may be an important quality for a bile acid-binding protein. These studies have revealed that a lipid molecule is bound by a hydrophobic pocket inside the protein in the presence of complexed water molecules. In the case of a bile acid, binding is based primarily on hydrophobic interactions with some hydrogen bonding for hydroxyl groups. The charge of the carboxyl acid or side chain is localized near or protruding from the opening and contacts an arginine residue.

Function – Although ILBP has been shown to bind both fatty acids and bile acids, a subsequent study has demonstrated that ILBP bound bile acids with a higher affinity (Gong *et al.*, 1994). ILBP is the only intracellular lipid-binding protein that is known to bind bile acids with any appreciable affinity. The only other protein in this family known to associate with bile acids is the promiscuously binding liver fatty acid-binding protein (L-FABP). However, L-FABP shows a relatively weak affinity for bile acids and this association is not thought to be physiologically relevant. Although *in vitro* studies have demonstrated the binding activity of ILBP, the physiological function(s) of the protein remains unknown. As intracellular lipid-binding proteins are purported intracellar lipid transporters, ILBP has long been hypothesized to be involved in the intracellular shuttling of bile acids. In support of this hypothesis, *in vitro* assay studies have demonstrated the bile acid binding

activity of ILBP (Sacchettini *et al.,* 1990; Gong *et al.,* 1994; Fujita *et al.,* 1995). However, there is still no direct evidence that this protein is physiologically relevant to the processes of bile acid transport or intracellular trafficking. As such, the mechanism for intracellular trafficking of bile acids in ileocytes remains a mystery. Recently, other functional possibilities have been hypothesized, including protection from bile acid cytotoxicity and the nuclear delivery of bile acids.

Regulation – Intestinal bile acid transport is upregulated during normal development in many species including humans. Associated with this phenomenon is the increase in ILBP protein and mRNA expression (Sacchettini *et al.,* 1990; Crossman *et al.,* 1994; Gong *et al.,* 1996). This ontogenic regulation has been shown to be caused by a post-weaning (third post-natal week) increase of circulating glucocorticoids (Hwang and Henning, 2000; Hwang and Henning, 2001). Evidence exists for the involvement of the FXR (Makishima *et al.,* 1999; Parks *et al.,* 1999; Wang *et al.,* 1999), a bile acid activated transcription factor, in the regulation of *ILBP* gene expression (Grober *et al.,* 1999; Chiang *et al.,* 2000). However, many studies have shown conflicting results with regards to the responsiveness of *ILBP* gene expression to a modulating presence of bile acids (Torchia *et al.,* 1996b; Arrese *et al.,* 1998; Coppola *et al.,* 1998; Grober *et al.,* 1999; Xu *et al.,* 2000). Since regulation of bile acid recovery in the ileum can vary between species (Shneider, 2001), one explanation may be the differential ability of FXR to interact with the ILBP promoter of the various species. It is generally accepted that, in mice, FXR activation promotes the transcription of ILBP. More recently, two links between cholesterol levels in the gut and ILBP mRNA expression have been established as an example of lipid cross-talk. Oxysterols have been shown to promote *ILBP* transcription via independent LXRα and the sterol regulatory binding protein 1C (SREBP1C) (Landrier *et al.,* 2002; Zaghini *et al.,* 2002; Landrier *et al.,* 2003). The fact

that sterol intracellular sensors FXR, LXR, and SREBP1C are involved in the control of ILBP gene expression strongly suggests a crucial role for ILBP in the ileum.

1.5.3 The Farnesoid X Reaceptor (FXR)

The discovery that bile acids are the natural ligands for the transcription factor FXR (NR1H4) has introduced the concept that bile acids have a role to play in gene regulation (Makishima *et al.*, 1999; Parks *et al.*, 1999; Wang *et al.*, 1999). FXR is a nuclear receptor which includes both non-steroidal and steroid receptors. Upon ligand biding, FXR will bind to a DNA *cis* element known as the FXR response element (FXRE). Non-steroidal receptors, like FXR, bind DNA as a heterodimer with the obligate partner 9-*cis* retinoic acid X receptor (RXR, NR2B1) (Mangelsdorf *et al.*, 1995). Rat FXR was originally cloned using PCR and degenerate primers corresponding to the semi-conserved DNA binding domain of nuclear receptors (Forman *et al.*, 1995). In this initial study, FXR was shown to be weakly activated by supraphysiological levels of the isoprenoid farnesol, which gave rise to the name of this nuclear receptor. More recent studies have shown that bile acids such as CA and CDCA bind to FXR *in vitro* and that this interaction occurs at physiological levels of the bile acids (EC_{50} of 10-15 μM) (Makishima *et al.*, 1999; Parks *et al.*, 1999; Wang *et al.*, 1999). This interaction was also shown to lead to the recruitment of coactivators to the ligand-bound FXR and that there is a subsequent increase in the transcription of target genes. *ILBP* was the first gene recognized as a target for FXR-mediated regulation but since then a handful of other genes has also been identified as FXR targets. However, most of these genes are expressed in the liver and the impact of FXR in ileocyte homeostasis remains unknown. RNA hybridization blot assays indicate that FXR transcripts in the

rat are mainly restricted to organs involved in steroid metabolism, namely the liver, kidney, adrenals, colon, and along the axis of the small intestine (Forman *et al.*, 1995).

Structure- FXR is a 54 kDa protein and is similar in structure to other members of the nuclear receptor 1 (NR1) family. It has highly conserved DNA-binding and ligand-binding domains (Forman *et al.*, 1995). Additionally, a RXR interaction domain has been identified. The FXR-RXR heterodimer typically binds to degenerates of an inverted repeat 1 (IR-1) element, meaning two inverted 6-base pair repeats with a 1-base pair spacing between the halfsites (Edwards *et al.*, 2002). This FXRE was later termed the bile acid response element (BARE) because of the nature of FXR ligand binding.

Function- Although FXR has a predominant role in regulating bile acid biosynthesis (section 1.3.1), there are limited described functions for FXR in the enterocyte. FXR is often pointed to as a positive regulator of ILBP transcription, a putative bile acid binder. Since ILBP is expressed so abundantly and is the only cytosolic protein in the enterocyte known to bind bile acids, it is deemed that the relationship between these two proteins must be significant in bile acid metabolism in the enterocyte and the body. However, since the physiological function of ILBP remains unknown, so does the relationship between these two proteins. FXR-deficient mice have been shown to abrogate the expression of ILBP (Kok *et al.*, 2003). This study suggests that FXR is necessary for the basal expression of ILBP. However, the purpose of this regulation remains a mystery.

FXR can act as both a repressor and activator of transcription. Activation of transcription of a target gene is accomplished as described above. Briefly, ligand-bound FXR heterodimerizes with RXR and binds to BARE to *cis* activate positive transcriptional regulation. As described in

section 1.3.1, FXR can also transcriptionally repress target genes through FXR-mediated induction of SHP. SHP is a nuclear receptor (NR0B2) that negatively interacts with other nuclear receptors and essential transcription activators (Goodwin *et al.*, 2000; Lee *et al.*, 2000; Lu *et al.*, 2000; del Castillo-Olivares and Gil, 2001; Zhang and Chiang, 2001). Mouse but not rat ASBT is regulated in this manner (Chen *et al.*, 2003). The mouse *ASBT* promoter binds and is activated by liver receptor homologue 1 (LRH1). SHP diminishes the activity of the mouse *ASBT* promoter and partially offsets its activation by LRH1. A similar FXR/SHP repression has been observed in the human *ASBT* promoter (Neimark *et al.*, 2004).

1.6 Gender Differences in Bile Acid Metabolism

This thesis is concerned with murine bile acid metabolism and an intracellular lipid-binding protein, both of which offer many examples of gender disparity. It is generally known, as reflected in Chapter 5, that gallbladder and tissue bile acid concentrations are generally higher in female mice compared to male mice when normalized to volume or tissue weight. Differing lipid concentrations in various tissues in male and female mice is mirrored by differing gender expression of genes involved in lipid metabolism. Female expression of bile acid metabolic-related genes can be seen in Figure 1-6 in comparison to males. Among biosynthetic genes (Figure 1-6A), it can be seen that female Cyp7a1 mRNA abundance is higher than males, which also matches the higher activity detected from activity assays (Dietschy and Turley, 2002). The depressed levels of Cyp8b1 in females, which controls the ratio of CA to β-MCA in the bile acid pool, is consistent with the previous observations that CA:β-MCA ratios are different between the genders. Also of particular interest are the lower levels of FXR mRNA in females. This suggests that the strength of responses to stresses

related to bile acid metabolism, such as fasting or illness, may also be gender specific. Among hepatic transporter genes (Figure 1-6B), it is easy to hypothesize that the increase in NTCP mRNA expression observed in females compared to males may be a response to compensate for higher bile acid concentrations in the portal circulation and to ensure the efficient removal of bile acids from blood by the liver. However, the purpose of lower ASBT and L-FABP mRNA abundance in the intestine of females compared to males is not as readily explained (Figure 1-6C). On the other hand, liver L-FABP expression in female rats is generally higher than in males (Ockner *et al.*, 1982). This coincides with the fact that hepatic free fatty acid uptake is also higher in female rats (Sorrentino *et al.*, 1992). Other examples of gender disparity in the expression of intracellular lipid-binding proteins include heart-FABP (Glatz *et al.*, 1985) and intestinal-FABP (Damcott *et al.*, 2003). From these studies, it can be inferred that, even though ILBP expression between the genders is similar (Figure 1-6C), studies employing the murine model must take in to account gender disparity generally and perhaps use it to better understand any differential effects observed in male and female mice.

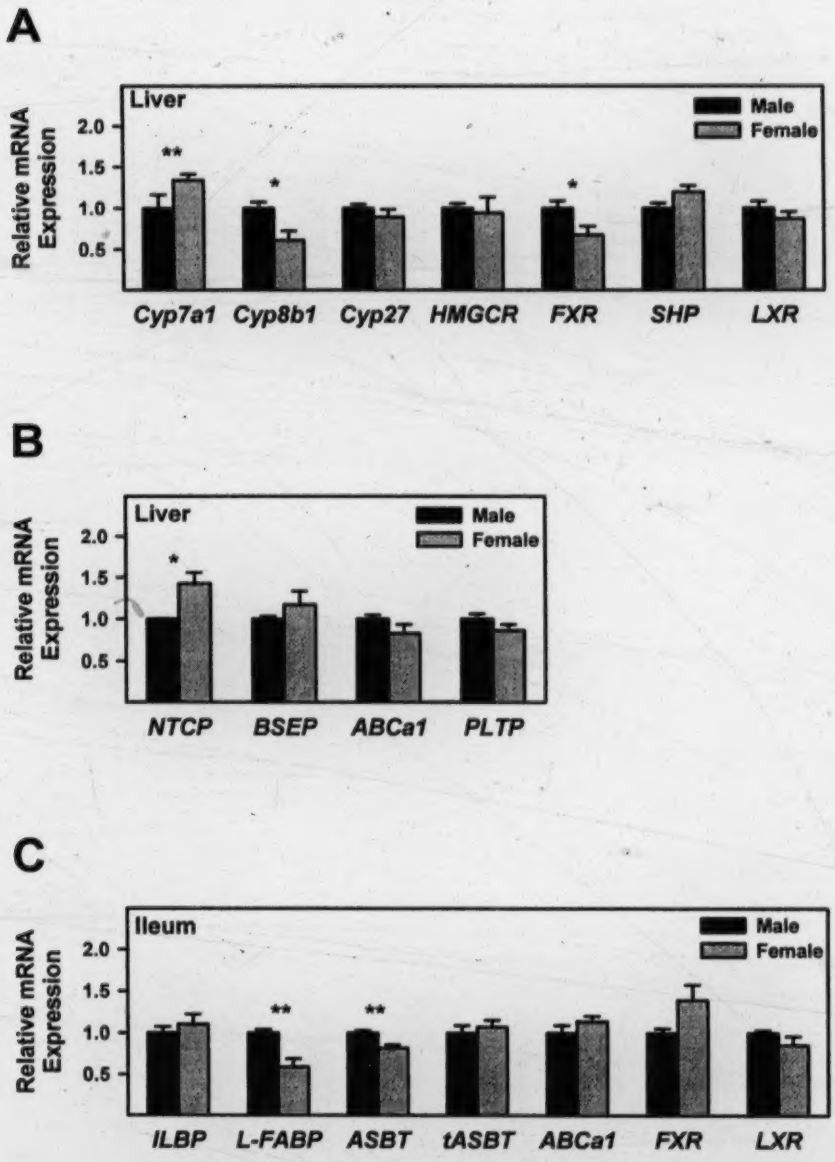

Figure 1-6. Comparison between genders of relative mRNA abundance of bile acid-related genes. Real time Q-PCR analysis of total RNA from C57BL/6J mice with primers designed for specific genes involved in hepatic sterol synthesis (A), hepatic lipid transport (B), and intestinal lipid metabolism (C). RNA extraction, reverse transcription, Q-PCR, and mathematical analysis are described in Chapter 2. Phosphollipid transfer protein (PLTP) is a known target of FXR-mediated regulation of transcriptional expression (Mak *et al.*, 2002). Data shown were determined using a comparative method standardized to 18S rRNA and shown as mean ± *SE*. Shown is an average of two experiments, $n = 6$ for all groups. *, $P < 0.05$; **, $P < 0.01$.

1.7 Thesis Question

ILBP has been proposed to act as a mediator of intracellular bile acid transport in the cytosol of ileocytes. Additionally, the physical interaction between ILBP and ASBT suggested a possible role for ILBP in bile acid transport. However, the precise binding preferences of this intracellular lipid-binding protein for various lipid species had not yet been characterized, nor was there an adequate method of bile acid separation, bile acid identification, or quantitation available in the laboratory. Published methods for quantitative bile acid analysis were found to be lacking in sensitivity or too costly to establish and maintain. Furthermore, the precise role of ILBP in the enterohepatic cycling of bile acids remained unknown. The overall goal of the work described in this thesis was to characterize the preferential lipid-binding abilities of ILBP and define the role of ILBP in cellular bile acid transport. To accomplish this, the following aims were proposed:

i. To develop a reliable, efficient, and cost-effective method for quantitative bile acid analysis.

ii. To determine the binding selectivity of recombinant murine ILBP toward both fatty acids and bile acids.

iii. To define the role of ILBP in the conservation of bile acids using a murine ILBP-deficient model.

1.8 References

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Chapter 2

General Methods

2.1 Chemicals

HPLC grade water, methanol, acetic acid, acetonitrile, and aqueous ammonia were purchased from commercial sources. The bile acid standards cholic acid (CA), chenodeoxycholic acid (CDCA), deoxycholic acid (DCA), ursodeoxycholic (UDCA), lithocholic acid (LCA), glycocholic acid (GCA), glycochenodeoxycholic acid (GCDCA), glycodeoxycholic (GDCA), glycoursodeoxycholic (GUDCA), glycolithocholic acid (GLCA), β-muricholic acid (β-MCA), murocholic acid (no abbreviation), nor-cholic acid (nor-CA), nor-deoxycholic acid (nor-DCA), taurocholic acid (TCA), taurodeoxycholic (TDCA), tauroursodeoxycholic (TUDCA), taurolithocholic acid (TLCA), taurochenodeoxycholic acid (TCDCA), and tauro-β-muricholic acid (β-TMCA) were purchased from Sigma-Aldrich (Oakville, ON, Canada) and Calbiochem (La Jolla, CA, USA). Bile acid standards were prepared in methanol and the final concentration of standards were calibrated with a 3α-steroid dehydrogenase-based colorimetric assay (bile acid diagnostic kit #450-A) purchased from Sigma-Aldrich Co. The fatty acid standards myristic acid (C14:0), palmitic acid (C16:0), stearic acid (C18:0), oleic acid (C18:1), linoleic acid (C18:2), α-linolenic acid (C18:3) and arachidonic acid (C20:4) were purchased from Sigma-Aldrich and Nu-chek Prep, Inc. (Elysian, MN, USA). [^{3}H]-taurocholic acid (3.47 Ci/mmol), [^{32}P]-α-dCTP (3000 Ci/mmol), [4-^{14}C]-cholesterol (10 mCi/mmol), [^{14}C]-3-hydroxy-3-methylglutaryl-CoA (10 mCi/mmol), and [^{14}C]-cholic acid (48.60 Ci/mmol) was purchased from Amersham Pharmacia Biotech (Baie d'Urfé, QC, Canada).

2.2 Molecular Biology

Standard molecular biology techniques were used for DNA transformation of bacteria, isolation of plasmid DNA, endonuclease digestion

of plasmid DNA, ligation of plasmid constructs, DNA amplification, and the generation of radiolabeled DNA probes (Ausubel *et al.*, 1989; Maniatis *et al.*, 1989).

2.3 Immunoblot Analysis

In preparation for immunoblotting of proteins, tissues and bacterial lysates were processed as described in individual chapters. Protein concentration from bacterial lysates or liver and intestinal homogenates were determined by the method Bradford (Bradford, 1976) using BSA as standard. Protein samples were processed and then resolved by SDS polyacrylamide gel electrophoresis (PAGE) by the method of Laemmeli (Laemmli, 1970) and transferred onto a PVDF (Millipore Ltd., Nepean, ON) membrane by electroblotting. Membranes were typically blocked 1 to 16 h with 5% skimmed milk powder in PBS containing 0.5% Tween 20 (TPBS), washed several times with TPBS, and incubated for 1 h with diluted primary antiserum in TPBS with 5% skimmed milk powder. Following primary antibody incubation, membranes were washed again with TPBS and incubated with appropriate horseradish peroxidase-conjugated secondary antiserum for 1 h. Membranes were extensively washed with TPBS before chemiluminescent detection using the ECL detection system (Amersham Corp., Baie d'Urfé, PQ). ILBP was detected using rabbit antiserum raised against the recombinant murine ILBP purified as detailed in Chapter 4 and published results (Labonte *et al.*, 2003).

2.4 RNA Blot Analysis

Total RNA was isolated using a one step guanidinium thiocyanate method (Chomczynski and Sacchi, 1987) and separated by electrophoresis

on a 1% agarose-formaldehyde gel (Maniatis *et al.*, 1989). The RNA was transferred onto a Hybond-N+ nylon membrane by capillary transfer (Southern, 1975), cross-linked using a Stratalinker device (Stratagene, La Jolla, Ca.) and probed with a radiolabeled cDNA probe labeled with [α-^{32}P]-dCTP prepared by the random priming method (Maniatis *et al.*, 1989) or the nick-translation method (Rigby *et al.*, 1977). The probe was hybridized in buffer containing 0.25 M sodium phosphate (pH 7.2), 7% SDS, 0.1% sodium pyrophosphate and 2 mM EDTA for 16 h at 60°C. Membranes were washed twice with 2X SSC containing 0.1% SDS for 15 min each at room temperature, twice with 1X SSC/0.1% SDS for 15 min each at room temperature, and once with 0.5 X SSC/0.1% SDS for 15 min at 60°C. Membranes were then exposed to x-ray film or a phosphorimaging screen. Phosphorimaging screens were scanned in a Fuji-X Bas 1000 phosphorimager. For re-probing, membranes were stripped of bound probe by incubating membranes in boiling water containing 0.1% SDS until the solution cooled to RT.

2.5 Real Time Q-PCR Analysis

Tissue samples for isolation of RNA were snap frozen in liquid nitrogen and stored at -80°C. Samples were homogenized and total RNA was isolated using the Tri Reagent method (Molecular Research Centre, Inc., Cincinnati, Ohio) according to manufacturer instructions. Integrity of the RNA was confirmed by agarose gel electrophoresis and RNA concentration was determined spectrophometrically. Single stranded cDNA was obtained from incubating 10 µg of total RNA for 1 h at 42°C with 120 U of avian myeloblastosis virus reverse transcriptase, 16 µl of 5-fold concentrated buffer, 100 U of RNase inhibitor, and 8 µl of dNTP mix (20 mM) (Roche, Mannheim, Germany).

Real-time comparative quantitation polymerase chain reaction (Q-PCR) was accomplished utilizing SYBR Green I (Molecular Probes, Burlington, ON) on a Corbett Research Rotor-Gene 3000 lightcycler (Montreal Biotech Inc., Kirkland, PQ) (Morrison *et al.,* 1998). SYBR Green I is a dye which fluoresces when bound to double-stranded DNA. PCR-based analysis of mRNA abundance was performed using the Corbett convection lightcycler with the following thermocycling profile: 95°C for 15 s, 60°C for 30 s, and 72°C for 30 s. PCR was accomplished utilizing Platinum Quantitative PCR SuperMix-UDG (Invitrogen, Burlington, ON) following manufacturer procedures. The cycle number at the threshold, after which the intensity of SYBR Green I fluorescence increases in a logarithmic linear fashion, was used to quantitate the PCR product utilizing several comparative mathematical models (Livak and Schmittgen, 2001; Pfaffl, 2001; Liu and Saint, 2002a, b). All reactions were optimized and then confirmed by agarose gel electrophoresis. Fluorescence due to amplicon production was differentiated from non-specific fluorescence by melting curve analysis. If necessary, a fourth detection step for each cycle at a higher temperature was added to avoid primer dimer formation. The primers utilized in each PCR reaction are described in Table 2-1. All annealing temperatures were 60°C except for 18S rRNA which was 58°C. ASBT has a splicing variant, truncated ASBT (tASBT), and PCR analysis must be able to differentiate between these two products. ASBT primers are located in exon 2, which is missing in tASBT; the antisense tASBT primer consists of two halves, one in exon 1, and the other in exon 3. Therefore, using the strategy from Dr. Kuiper's lab (Hulzebos *et al.,* 2003), this PCR setup can differentiate between the functionally different ASBT and tASBT. All expression data were subsequently standardized for 18S rRNA, which was analyzed separately.

Table 2-1. Sense and antisense oligonucleotides used for Q-PCR analysis.

Target cDNA	Primer Sequences	Size (bp)	Detection Temperature
18S rRNA	5'-TTG ACG GAA GGG CAC CAC CAG-3' 5'-GCA CCA CCA CCC ACG GAA TCG-3'	89	72°C
ABCa1	5'-CCC AGA GCA AAA AGC GAC TC-3' 5'-GGT CAT CAT CAC TTT GGT CCT TG-3'	102	72°C
ASBT	5'-ACC ACT TGC TCC ACA CTG CTT-3' 5'-CGT TCC CGA GTC AAC CCA CAT-3'	84	72°C
BSEP	5'-CTG CCA AGG ATG CTA ATG CA-3' 5'-CGA TGG CTA CCC TTT GCT TCT-3'	103	76°C
Cyp7a1	5'-CAG GGA GAT GCT CTG TGT TCA-3' 5'-AGG CAT ACA TCC CTT CCG TGA-3'	121	77°C
Cyp8b1	5'-TTC TCT TCC CCA GGT TTG TCT-3' 5'-TTT TCC AGG TTT TGC TCC AC-3'	112	72°C
Cyp27	5'-GCC TTG CAC AAG GAA GTG ACT-3' 5'-CGC AGG GTC TCC TTA ATC ACA-3'	107	72°C
FXR	5'-GTG TAA GAA CGG GGG CAA C-3' 5'-ACA AAC ATT CAG CCA ACA TCC-3'	107	80°C
GAPDH	5'-GAG CCA AAC GGG TCA TCA TC-3' 5'-CAT CAC GCC ACA GCT TTC CA-3'	95	72°C
HMGCR	5'-CCG GCA ACA ACA AGA TCT GTG-3' 5'-ATG TAC AGG ATG GCG ATG CA-3'	115	72°C
ILBP	5'-CAG GAC GGA CAG GAC TTC AC-3' 5'-TGG TCT GCA TTT CAC ATT CTT T-3'	97	72°C
L-FABP	5'-AAA TCG TGC ATG AAG GGA AG-3' 5'-GTC TCC AGT TCG CAC TCC TC-3'	100	72°C
LXRα	5'-GCT CTG CTC ATT GCC ATC AG-3'. 5'-TGT TGC AGC CTC TCT ACT TGG A-3'	80	78°C
NTCP	5'-ATG ACC ACC TGC TCC AGC TT-3' 5'-GCC TTT GTA GGG CAC CTT GT-3'	111	72°C
PLTP	5'-TCA GTC TGC GCT GGA GTC TCT-3' 5'-AAG GCA TCA CTC CGA TTT GC-3'	80	72°C
SHP	5'-AAG GGC ACG ATC CTC TTC AA-3' 5'-CTG TTG CAG GTG TGC GAT GT-3'	72	72°C
tASBT	5'-AGG CTG TAG TGG TGC TAA TTA TG-3' 5'-CAG AGA AAT ACC TGA GGT CCA T-3'	102	72°C

Comparative quantitative real-time PCR was performed utilizing SYBR green I. Size refers to the base pair (bp) length of the amplified products. Detection temperature refers to the temperature utilized to detect DNA amplification after each extension cycle. If 72°C then readings were made immediately following extension, other temperatures mean the introduction of a fourth reading step at a higher temperature to reduce dimer primer interference.

2.6 *Plasma Lipid Analysis*

Plasma cholesterol and triglyceride was measured with the Infinity Cholesterol Reagent and the Serum Triglyceride Determination kit (Sigma Diagnostics, Inc., St. Louis, MO), respectively, using manufacturer's protocol. To measure lipoprotein lipid profiles, equal amounts of all plasma samples from a treatment group were mixed. Lipoprotein lipid profiles were obtained from these pooled samples by high performance gel filtration chromatography as previously described (Kieft *et al.*, 1991). Briefly, plasma lipoproteins were separated by size exclusion chromatography on a high performance liquid chromatography system (Beckman Instruments Canada, Inc., Mississauga, ON) fitted with a Superose 6 gel filtration column (Amersham Pharmacia Biotech, Inc., Baie d'Urfé, Quebec). Lipoprotein fractions were then mixed with the Infinity Cholesterol Reagent or Serum Triglyceride Reagent using a post-column T-connector and passed through a 12-inch line heater set at 37°C. Reaction products were monitored in real-time either at 500 nm for cholesterol or 540 nm for triglyceride using a visible-light detector (Beckman).

2.7 *Plotting and Statistics*

All values represent mean ± standard error for the number of animals or experiments indicated. A two-tailed Student's t-test assuming equal variance or a single-factor analysis of variance (ANOVA) followed by least significant difference post hoc test were used for comparison of data from different experimental conditions (Altman, 1991). Level of significance was set at $P < 0.05$ (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$). Plotting was performed using SigmaPlot v. 8 for Windows software (SPSS Inc.). Analysis was performed

using Microsoft Office Excel 2003 (Microsoft Corp.) or SAS/Stat v. 8 (SAS Institue Inc.).

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Chapter 3

Quantitation of Bile Acids Using an Isocratic Solvent System for HPLC

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3.1 Introduction

As mentioned in Chapter 1, bile acids are amphipathic molecules that are produced in the liver and show diversity in the state of hydroxylation of the steroid moiety (Russell and Setchell, 1992; Hofmann, 1999). Prior to secretion into bile, the primary bile acids are converted into N-acyl conjugates of either glycine or taurine. Conjugation increases the acidity of bile acids and enables these molecules to form bile salts readily. In the intestine, bacterial enzymes modify these compounds by removal of the N-acyl conjugates and dehydroxylation of the steroid moiety. As a result, the metabolism of bile acids produces a group of sterol acids that differ greatly in hydrophobicity, acidity, and size.

The studies performed in chapters 4 and 5 of this thesis require an effective and facile detection method for both synthetic and naturally occurring bile acids. Examination of the published methods for chromatographic analysis of bile acids available revealed a common problem. The major impediment to a convenient and informative analysis of bile acids has been the difficulty of resolving a wide variety of bile acid species that differ in hydrophobicity and other physicochemical properties. For example, bile acids exhibit a wide variance in capacity to absorb UV light, which makes detection of these molecules by UV spectrometry inconvenient (Rossi *et al.,* 1987). Furthermore, UV light absorption detection of bile acids has also displayed poor sensitivity. Of published methodologies, those employing high performance liquid chromatography (HPLC) or gas chromatography (GC) in combination with mass spectrometry (MS) have proven powerful in addressing the problems of bile acid diversity (Setchell *et al.,* 1983; Perwaiz *et al.,* 2001). GC-MS techniques are sensitive but require that bile acids be highly purified and derivatized before analysis. Liquid chromatography-electrospray tandem mass spectrometry is efficient and

rapid for the detection of bile acids, but is costly to establish and maintain. Given the variety and number of samples to be measured in this thesis and the nature of the experiments, it became necessary to generate a new method for the resolution and detection of bile acids, one which displayed enhanced versatility, and sensitivity, and that could be run in the laboratory at low cost.

Previously developed methods based on HPLC coupled to an evaporative light scattering detector (ELSD) permitted the resolution of both conjugated and unconjugated bile acids (Novakovic *et al.*, 1998; Roda *et al.*, 1998; Criado *et al.*, 2003). Of the methods available, these were the most promising in filling the criteria listed above. In an ELSD, the mobile phase and samples are atomized so that particles passing through a laser can be detected. However, the use of a solvent gradient to resolve bile acids was problematic since every elution peak needs to be calibrated due to the sensitivity of the ELSD to solvent composition. This chapter describes a simple and convenient HPLC method that employs a single isocratic solvent system for the separation and quantitation of both conjugated and unconjugated bile acid species. The HPLC-ELSD detection of bile acids described in this chapter is more sensitive and resolves a wider range of bile acid species more quickly than traditional methods.

3.2 Methods

3.2.1 Animals and diets

Female inbred mice (C57BL/6J and 129/J) and female outbred CD-1 mice were purchased from Jackson Laboratory (Bar Harbor, ME). Animals

were 10 weeks old and maintained in a reverse 12 h light and 12 h dark cycle and given free access to chow and water for two weeks. Animals were fed rodent chow (#5001, Ralston Purina, St Louis, MO) and fasted overnight prior to sacrifice. For the diet study, five C57BL/6J mice were fed rodent chow supplemented with 0.5% TCA and another group of five mice were fed rodent chow only. At sacrifice, gallbladders were collected and crude bile was collected. For the study comparing the C57BL/6J, CD-1, and 129/J mice, six animals of each group were maintained on normal rodent chow for 2 weeks. After an overnight fast, mice were anesthetized and exsanguinated, and their livers, gallbladders, and small intestines were collected.

3.2.2 Bile and bile acid standard sample preparation

Gallbladder bile acids were prepared for analysis as described previously (Stolyhwo *et al.*, 1983). Bile samples were diluted with 5 volumes of absolute methanol to precipitate proteins. Samples were then centrifuged at 20,000 x g for 10 min at ambient temperature and supernatant was further diluted (1/20) in methanol that included nor-DCA (146 μM) as an internal reference for peak identification. The diluted bile sample (6 μl) was analyzed by HPLC-ELSD.

In vitro bile acid binding assays were performed to assess the association of bile acids with delipidated recombinant murine ILBP. ILBP (23.4 nmol) was incubated with a five-fold molar excess of a TCDCA and CDCA (approximately 1:2) mixture in a total volume of 400 μl at 37 °C for 1 h. Unbound bile acids were then removed from protein-bile acid complexes by size exclusion gel filtration using a Sephadex G25 column (Amersham Pharmacia Biotech Inc., Baie d'Urfé, PQ). Fractions containing ILBP were collected and pooled. The bound bile acids were extracted by C18/14 Spe-

ed SPE cartridges from Applied Separations (Allentown, PA, USA). The cartridges were conditioned before usage according to the manufacturer's instructions. The samples (2.5 ml) were then applied to cartridges and washed with distilled water (8 ml) before elution with methanol (4 ml). The eluant was filtered through a 0.22 μm Millex-GS filter (Millipore, Bedford, MA), dried down under nitrogen gas, and dissolved in 100 μl of methanol before analysis by HPLC-ELSD. The column does not retain salt and other highly hydrophilic compounds in our solvent system. A minimum volume of methanol was used to elute bile acids in order to minimize the amount of impurities, which are detected as the unretained peak.

3.2.3 Chromatographic instrumentation

Bile acids were analyzed using an HPLC gold Nouveau system from Beckman Coulter Inc. (Mississauga, Ontario). The instrument was equipped with a Luna C_{18} analytical column (5 μm particle size, 25 cm x 4.6 mm I.D.) fitted with a C_{18} guard column (4 mm x 3.0 mm I.D.) (Phenomenex U.S.A., Torrence, CA). An Altech ELSD 2000 (Altech Corp., Deerfield, IL) detector was used for detection of bile acids.

3.2.4 Solvent system and detector configuration

The composition of the mobile phase was determined using previously published bile acid HPLC methods and from the chemical nature of bile acids; as well, numerous optimization experiments performed by both Enrique Torchia and me. Optimization for both elution time and resolution was achieved by testing solvent ratios, salt content, and mobile phase pH. The final solvent system was composed of methanol:acetonitrile:water

(53:23:24, v:v:v) containing 30 mM ammonium acetate adjusted to pH 5.6 with acetic acid. The mobile phase was filtered through PTFE 0.45 μM filters (Pall Geldman, Mississauga, ON). Chromatography was performed at a flow rate of 0.7 ml/min at ambient temperature. ELSD response was optimized by testing peak areas resulting from various gas flow and drift tube temperatures. The ELSD was used with a gas flow of 1.3 L/min and a drift tube temperature set at 93°C. Peaks were identified based on their retention times as compared to authentic bile acid standards and the areas under the peaks were quantitated by an external standardization method. Prior to the sample run, the column was conditioned with the running solvent at the working flow rate (0.7 ml/min) for a minimum of 3 h to ensure reproducibility. The column was purged with 100% methanol at the same flow rate for 1 h prior to storage.

3.3 Results

3.3.1 Method Development

A single isocratic solvent system was developed that can rapidly resolve both conjugated and unconjugated bile acids simultaneously. The use of an ELSD in bile acid analysis is an improvement over other detectors because it does not rely on the optical properties of bile acids or the need for post- or pre- column derivatization associated with colorimetric assays (Stolyhwo *et al.*, 1983; Tazawa *et al.*, 1984). Previous HPLC-ELSD strategies have employed solvent systems composed of methanol and ammonium acetate with an elution gradient of the methanol component to achieve complete separation of both conjugated and unconjugated bile acids (Roda *et al.*, 1992; Roda *et al.*, 1995). However, because the ELSD is

sensitive to solvent composition, gradient protocols increase baseline drift and change the sensitivity of the detector to the individual bile acids. The polar solvent acetonitrile was introduced to the solvent system to eliminate the need for an elution gradient, thus reducing excessive solvent waste. Addition of acetonitrile decreased the retention time of unconjugated bile acids and prevented excessive tailing of late-eluting peaks. The presence of ammonium acetate increased the ionic strength of the mobile phase and allowed hydrophilic bile acids such as β-TMCA to be separated from the unretained peak (Table 3-1 and Figure 3-1A). The content of acetonitrile and ammonium acetate in the mobile phase was titrated to give optimal separation of both conjugated and unconjugated bile acid species. The final composition of the mobile phase was methanol/acetonitrile/water (53:23:24, v/v) containing 30 mM ammonium acetate adjusted to pH 5.6. Table 3-1 summarizes the retention times, capacity, and separation ratios for a variety of human and mouse bile acid standards utilizing the method described.

Table 3-1. Retention times of selected bile acid species

Peak No.	Bile acid species	MW	RT (min)	k'	α
	Unconjugated bile acids				
1	3α,6β,7β-trihydroxy-5β-cholanoic acid (β-MCA)	408.6	9.18	1.59	0.35
2	3α,7α,12α-trihydroxy-5β-cholanoic acid (CA)	408.6	15.43	3.37	0.75
3	3α,7α-dihydroxy-5β-cholanoic acid (CDCA)	392.6	27.40	6.75	1.49
4	3α,12α-dihydroxy-5β-cholanoic acid (DCA)	392.6	29.99	7.48	1.66
5	3α,7β-dihydroxy-5β-cholanoic acid (UDCA)	392.6	11.53	2.26	0.50
6	3α,7α,12α-trihydroxy-23-nor-5β-cholanoic acid (nor-CA)	394.6	10.03	1.84	0.41
7	3α,12α-dihydroxy-23-nor-5β-cholanoic acid (nor-DCA)	378.6	19.50	4.52	1.00
8	3α-hydroxy-5β-cholanoic acid (LCA)	376.6	55.23	14.63	3.24
	2-aminoethanesulfonic acid (taurine) conjugates				
9	β-TMCA	515.7	5.00	0.42	0.09
10	TCA	515.7	7.63	1.16	0.26
11	TCDCA	499.7	10.80	2.06	0.46
12	TDCA	499.7	12.20	2.45	0.54
13	TUDCA	499.7	5.97	0.69	0.15
14	TLCA	483.7	18.23	4.16	0.92
	Aminoacetic acid (glycine) conjugates				
15	GCA	465.6	8.75	1.48	0.33
16	GCDCA	449.6	12.85	2.64	0.58
17	GDCA	449.6	14.63	3.14	0.70
18	GUDCA	449.6	6.62	0.87	0.19
19	GLCA	433.6	22.58	5.39	1.19

Molecular weights (MW) are based on the chemical formula of the acid form and Retention Time (RT) is the time in min to elute the bile acid species. The capacity ratios (k') and separation ratios (α) were calculated as previously described (Gilbert, 1987). The reference compound used for the separation ratios was nor-DCA.

Figure 3-1A depicts the typical separation of a mixture of conjugated bile acids using the developed system. In rodents and humans, conjugated bile acids predominate in the enterohepatic pool of bile acids (Schwarz *et al.*, 1997; Turley *et al.*, 1998). Separation of these species of bile acids was complete by 30 min. Figure 3-1B illustrates the separation of a mixture of unconjugated bile acids. Both conjugated and unconjugated bile acids eluted from the column based on their hydrophobicity. For example, retention times

of 6β-hydroxylated bile acids (i.e., β-TMCA, β-MCA), which are present in mouse bile (Ishibashi *et al.*, 1996), were shorter compared to other tri-hydroxy bile acids such as TCA or CA (Figure 3-1 and Table 3-1). Taurine-conjugated bile acids eluted earlier than corresponding glycine-conjugated bile acids, emphasizing an important difference between taurine and glycine conjugation on determining bile acid hydrophobicity. To further illustrate the versatility of the developed method, a sample was injected containing a complex mixture representing 14 human bile acid species. Separation of conjugated and unconjugated bile acids was achieved in less than 35 min with the exception of LCA, a mono-hydroxylated and highly hydrophobic bile acid, which eluted at 55 min after injection (Figure 3-2). Previously published isocratic solvent systems separated only a few conjugated or unconjugated bile acids on a single run (Rajevic and Betto, 1998). I observed no degradation of signal or peak shape over a period of 2 years of continuous use with the occasional cleaning of the ELSD ionization tube. Additionally, retention times for the various bile acids were very reproducible between different runs and between long periods of column inactivity.

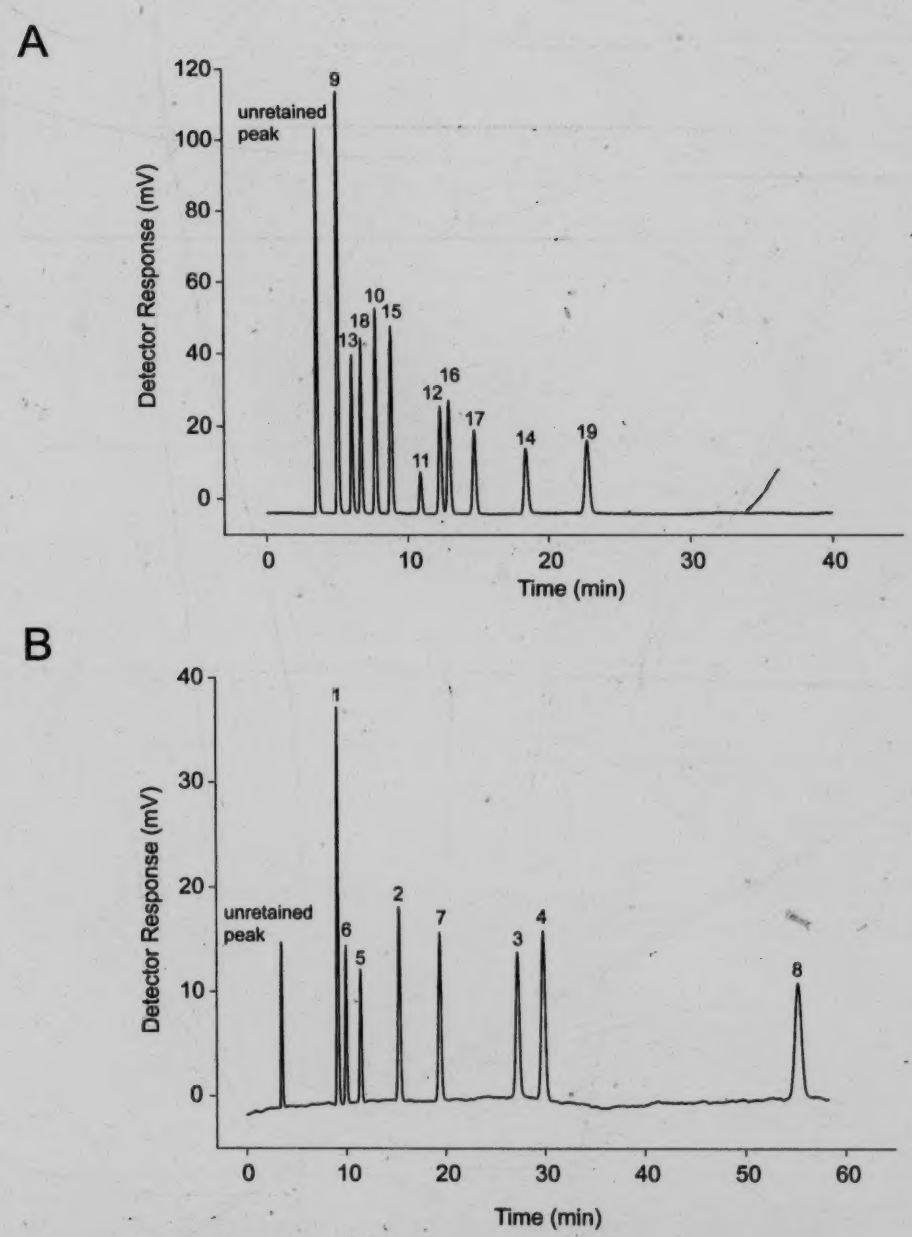

Figure 3-1. The typical separation of bile acids using the developed solvent system. A. Separation of conjugated bile acids. Note the complete separation of these species by 30 min. Order of eluting bile acids: β-TMCA (peak 9), TUDCA (peak 13), GUDCA (peak 18), TCA (peak 10), GCA (peak 15), TCDCA (peak 12), GCDCA (peak 16), GDCA (peak 17), TLCA (peak 14), and GLCA (peak19). B. Separation of unconjugated bile acids. Order of elution: β-MCA (peak 1), nor-CA (peak 6), UDCA (peak 5), CA (peak 2), nor-DCA (peak 7), CDCA (peak 3), DCA (peak 4), and LCA (peak 8).

Calibration curves were generated for various bile acid species by determining detector response to various amounts of injected bile acids. Amounts ranging from 0.08 to 10 nmol were injected and corresponding peak areas were tabulated. As shown in Figure 3-3, the amount of bile acid injected was logarithmically proportional to the peak area. According to ELSD theory, the response area of a solute is not linear with that of the sample mass (Lafosse *et al.*, 1987). The level of sensitivity achieved by the HPLC-ELSD system described above represents an improvement in the HPLC analysis of bile acids due to the minimization of degradation of signal to noise ratio associated with gradient systems.

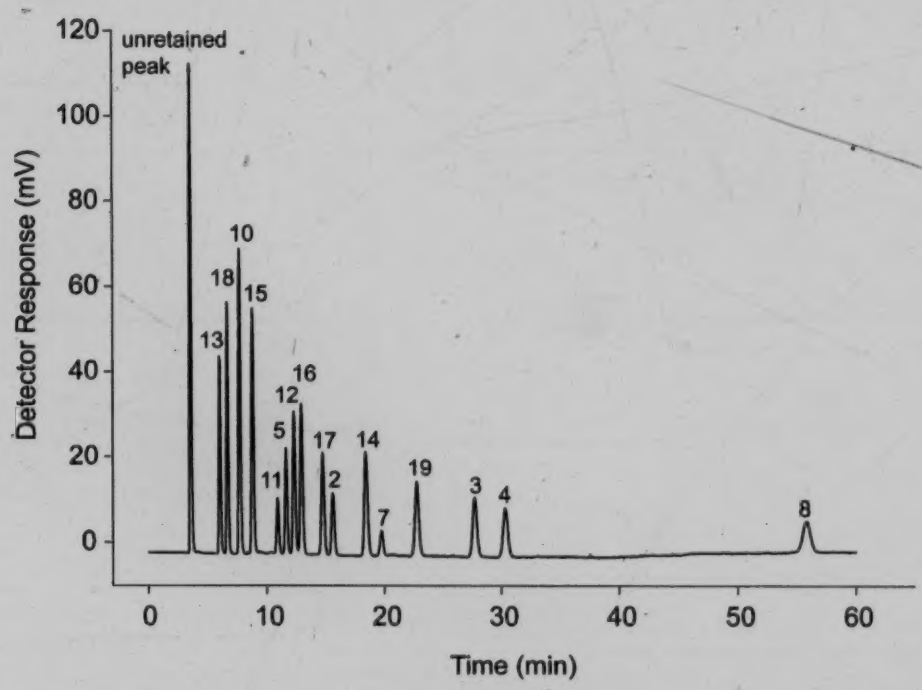

Figure 3-2. Separation of a complex mixture composed of 14 bile acids using the developed method. Order of elution: TUDCA (peak 13), GUDCA (peak 18), TCA (peak 10), GCA (peak 15), TCDCA (peak 11), UDCA (peak 5), TDCA (peak 12), GCDCA (peak 16), GDCA (peak 17), CA (peak 2), TLCA (peak 14), nor-DCA (peak 7), CDCA (peak 3), DCA (peak 4), and LCA (peak 8).

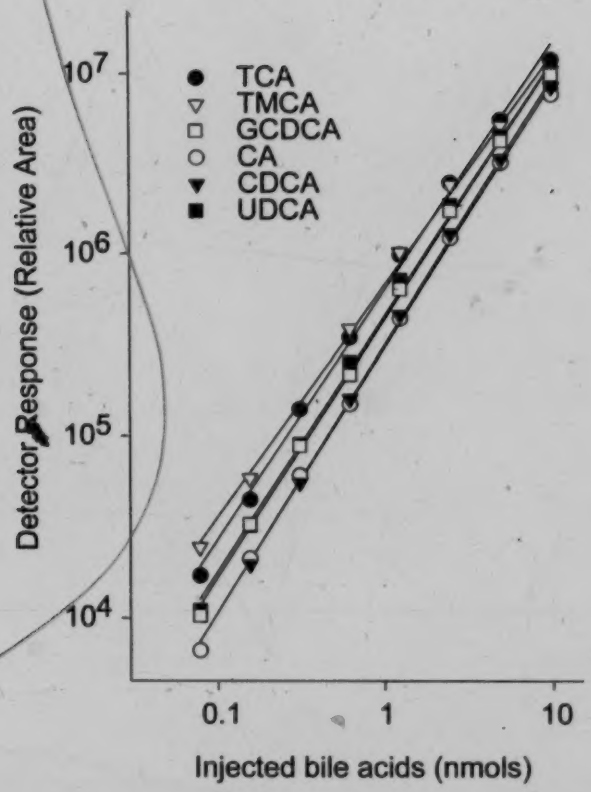

Figure 3-3. Calibration curves for selected bile acids. Amounts ranging from 0.08 to 10 nmol were injected for each bile acid and the resulting peak areas were tabulated. Coefficients of the regression lines ranged from 0.991 to 0.998.

3.3.2 Application of the methodology

The developed method was used to measure the amount of bile acids bound by the ILBP. ILBP is a known bile acid binder (Sacchettini *et al.*, 1990). As shown in Figure 3-4, the analysis of the bound bile acids after crude extraction was not affected by the residual ILBP remaining in the sample. The bile acid peaks were well separated from sample impurities,

which eluted as the unretained peak. The average area of this unretained peak was equivalent (98.6% ± 2.6%, mean ± S.D., n = 4) to that observed in reactions that contained ILBP alone. Note the reproducibility of the detection and quantitation of bound bile acids by the method (Figure 3-4, inset).

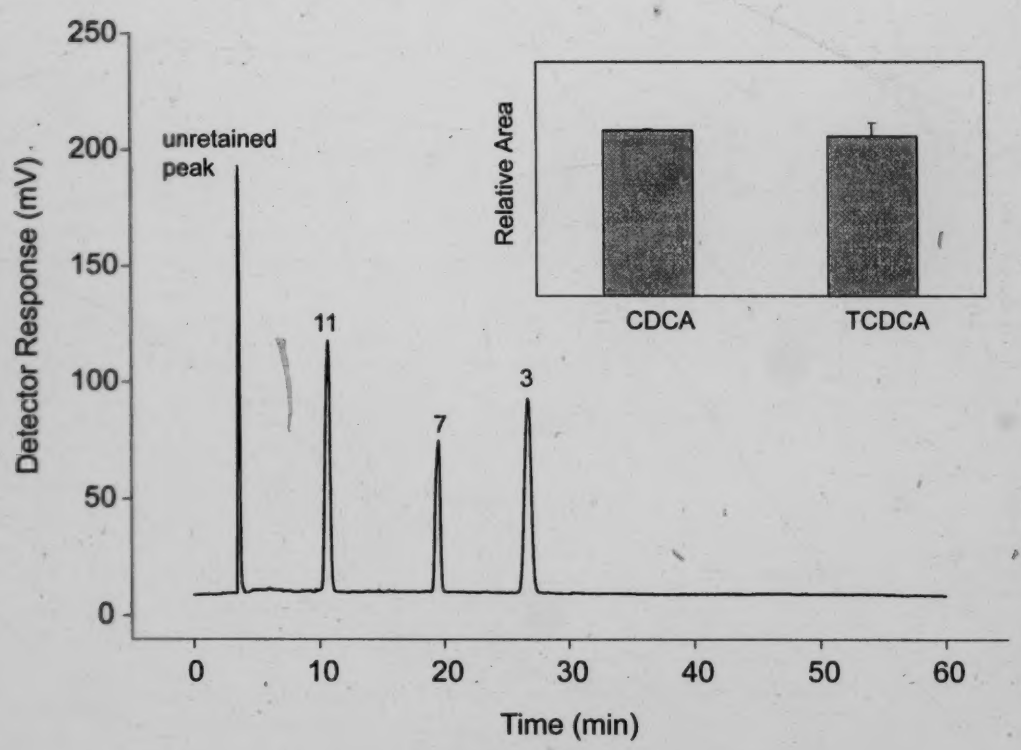

Figure 3-4. A representative profile profile of TCDCA and CDCA retained by recombinant ILBP. The peak numbers (11, 7, and 3) correspond to bile acid standards TCDCA, nor-DCA, and CDCA, respectively. The peak areas were determined relative to the nor-DCA internal reference standard, which was added prior to crude extraction. The inset shows a bar graph of areas determined from independent runs (mean ± SD, n = 6).

To illustrate the versatility of the developed method, the different species of bile acids and their relative levels in crude bile extracts were characterized. As shown in Figure 3-5, bile acids were easily detectable without interference from other biliary lipids such as cholesterol and phospholipids. No loss of signal or interference was observed even after 5 consecutive injections of bile samples. More recent applications of this method have seen no interference or loss of signal after 20 consecutive injections. The predominant bile acid species in mice fed a chow diet were taurine conjugates of cholic acid and β-muricholic acid (Figure 3-5A). The average ratio of TCA/TMCA in these mice was close to unity (0.97 ± 0.14, n=6), but increased dramatically in TCA-fed mice (21.82 ± 9.07, n = 5). Accordingly, TCA became the predominant bile acid in TCA-fed mice (Figure 3-5B). The concentrations of total bile acids in the bile of chow-fed animals as determined by the developed method (197 - 216 mmol/L) were comparable to values determined with the commercial bile acid diagnostic kit (123 - 294 mmol/L). These values are consistent with those reported previously for mice (175-206 mmol/L) (Dueland *et al.*, 1993; Jolly *et al.*, 1999).

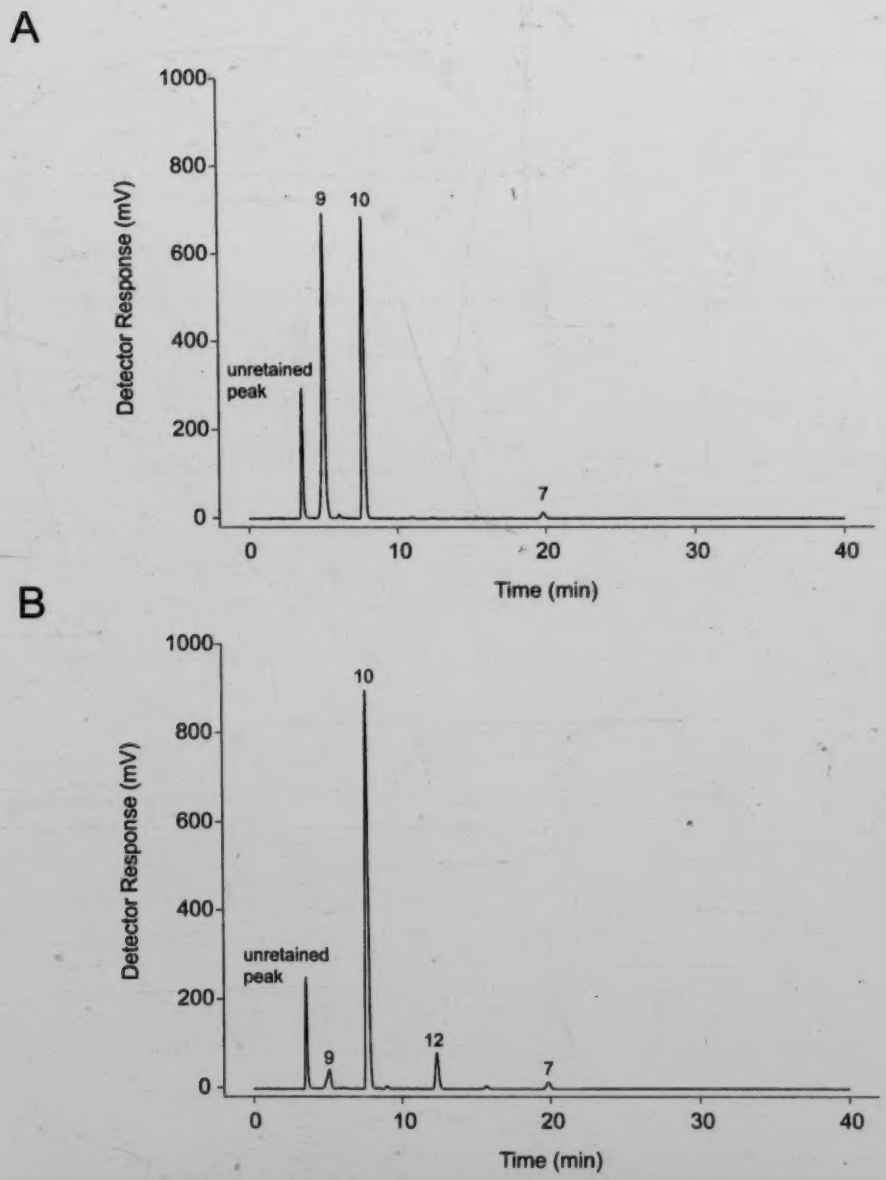

Figure 3-5. Profiles of biliary bile acids in mice fed a standard rodent chow diet (A) or the standard chow diet supplemented with TCA (B). Note the comparable amounts of TMCA (peak 9) and TCA (peak 10) in the bile of mice fed the chow diet. Upon use of the TCA-supplemented diet, TCA became the predominant bile acid found in bile. Note the appearance of TDCA (peak 12) in TCA fed mice. TDCA is a degradation product of TCA and is carried out by enteric bacteria. Nor-DCA (peak 7) was added to the bile samples prior to analysis. These profiles are representative of at least five animals for each condition. Mouse feeding, bile collection, and bile acid analysis for this figure was performed by Enrique Torchia.

To demonstrate the sensitivity of the developed method, crude bile extracts from three strains of female mice were analyzed for subtle changes in bile acid species composition. Genetic variation between murine strains allow for differences in phenotype that may be, at times, difficult to detect. Table 3-2 displays some physical characteristics of two inbred strains, C57BL/6J and 129/J, and an outbred strain, CD-1. The CD-1 strain is a somewhat larger mouse and has a higher body weight than the other two strains. However, C57BL/6J mice have more in common with CD-1 mice in the measured parameters than with the 129/J strain. While the liver weights are similar amongst all three strains when normalized to body weight, 129/J displays a longer intestine and increased gallbladder bile volume compared to C57BL/6J and CD-1 when normalized in the same manner. Intestine length can affect dietary lipid absorption, as can the volume of bile applied to emulsify lipids. These data suggest that 129/J may have a small variation in lipid absorption strategy compared to C57BL/6J and CD-1. This is supported by previous mouse strain studies which revealed that intestinal cholesterol absorption is higher in 129/J mice compared to C57BL/6 mice (Jolly *et al.*, 1999; Schwarz *et al.*, 2001a, b). Examination of the plasma cholesterol provided further evidence of a slightly difference in lipid metabolism in the three strains of mice. Total plasma cholesterol (Figure 3-6A) in 129/J was significantly higher than C57BL/6J, which is in agreement with the mouse strain study performed by the Jolly group. Further, examination of the lipoprotein cholesterol profile reveals HDL cholesterol in 129/J to be the highest of the three strains (Figure 3-6B). These profiles revealed that CD-1 was also elevated in cholesterol in comparison to C57BL/6J. It is possible that increased intestine length and bile volume in 129/J could account for the increased intestinal cholesterol absorption and in elevated plasma cholesterol levels compared to C57BL/6J. However, it should be noted that many other different parameters resulting from any number of genetic

differences between these two strains could achieve the same result. Other factors could include, but are not limited to, differences in intestinal villi length, lipoprotein secretion, lipoprotein clearance, or bile formation and processing.

Table 3-2. Body weight, liver weight, intestine length, and gallbladder bile volume in three strains of mice.

Parameter	C57BL/6J	CD-1	129/J
Body Weight (g)	20.0 ± 0.7	28.6 ± 0.6***	19.9 ± 0.3
Intestine Length (cm)	35.0 ± 0.8	48.2 ± 0.6	40.6 ± 0.4
Liver Weight (g)	0.70 ± 0.04	0.92 ± 0.02	0.63 ± 0.01
Bile Volume (µl)	15.6 ± 3.19	26.0 ± 3.5	37.8 ± 4.1
Ratios			
Intestine Length / BW	1.76 ± 0.03	1.69 ± 0.06	2.04 ± 0.04***
Liver Weight / BW	0.035 ± 0.001	0.032 ± 0.001	0.032 ± 0.002
Bile Volume / BW	0.80 ± 0.19	0.91 ± 0.14	1.87 ± 0.17***

Values represent means ± SE of 6 animals. Mice were 10 week old adult females fed a basal rodent chow diet and fasted before sacrifice. Bile volume refers to the volume of bile in the gallbladder at time of sacrifice. BW, Body weight. Differences among groups of mouse strains were assessed for statistical significance by using single-factor analysis of variance (ANOVA). This was followed by least significant difference test as a post hoc test. *** Significantly different ($P < 0.001$) from corresponding values for C57BL/6J mice.

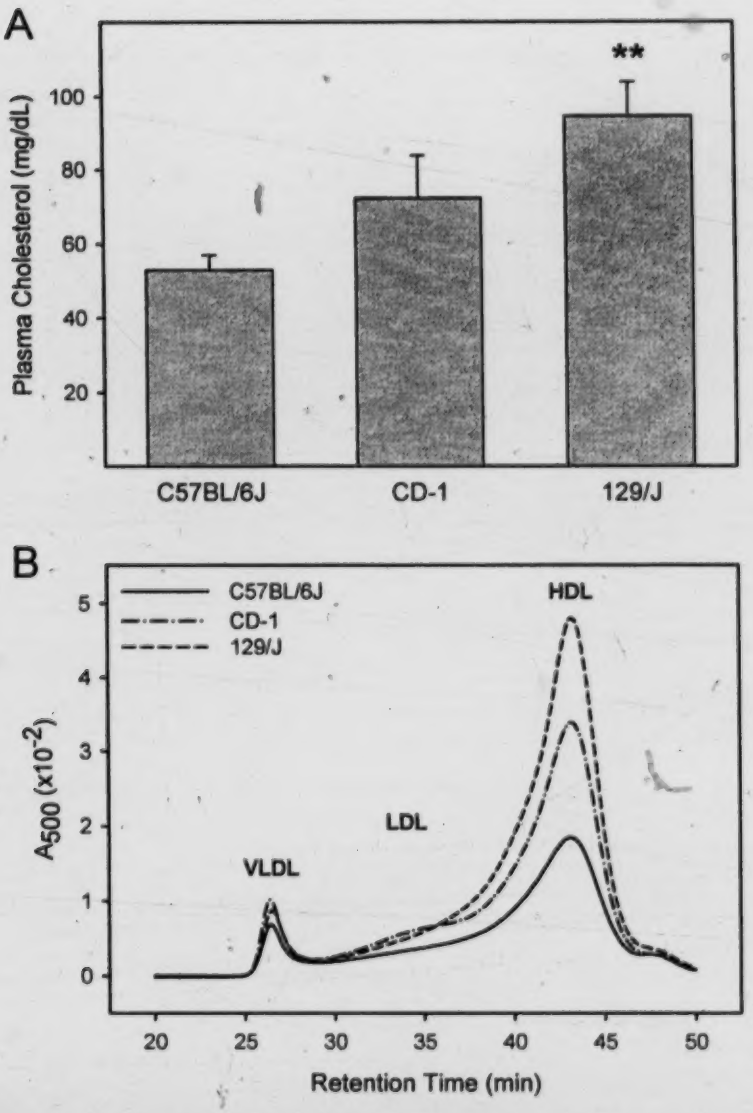

Figure 3-6. Plasma cholesterol analysis in C57BL/6J, CD-1, and 129/J mice. (A) Total cholesterol was measured in fresh plasma from each group (n=6). The data shown are mean ± SE. Differences among groups of mouse strains were assessed for statistical significance by using single-factor analysis of variance (ANOVA). This was followed by least significant difference test as a post hoc test. ** Significantly different ($P < 0.01$) from corresponding values for C57BL/6J mice. (B) Distribution of plasma lipoprotein cholesterol particle size in three mouse strains. The data shown were produced by pooling equal amounts of plasma for strain-matched animals prior to analysis by fast protein liquid chromatography.

Another factor that could account for differences in dietary lipid absorption between strains of mice is the composition of bile and the species of bile acids present. As Figure 3-5 illustrates, the two major bile acid species in the bile of mice are CA and β-MCA. These two species of bile acids represent over 95% of the bile acid pool in mice. Figure 3-7 shows that while the C57BL/6J bile acid pool is made up of equal amounts of CA and β-MCA, 129/J had a significantly higher amount of CA present. This result is similar to a previously published report comparing these two strains (Jolly *et al.*, 1999). Since CA is linked with more effective intestinal cholesterol absorption compared to β-MCA (Wang *et al.*, 2003), 129J should have the advantage in cholesterol absorption given that bile acid concentration in the bile of all three strains was similar. Additionally, there is more total bile acid ready to be utilized by the 129/J mice compared to the other two strains due to increased bile volume in the gallbladder (Table 3-2). These conditions would also favour cholesterol absorption in the 129/J intestine. The developed method was, therefore, not only able to rapidly determine total bile acid concentration but was also sensitive enough to detect differences in bile acid speciation in the bile among three strains of mice.

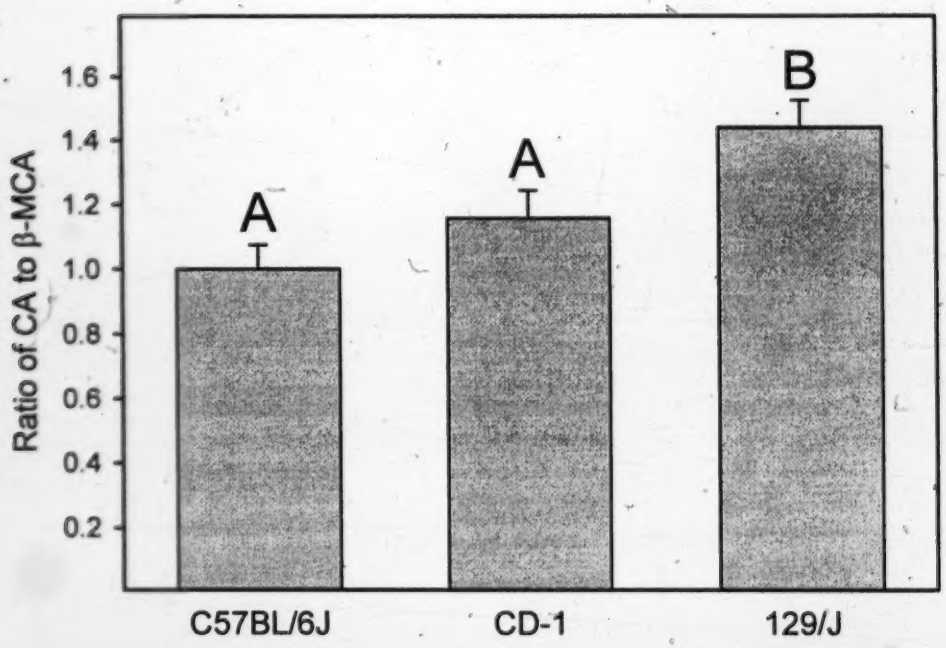

Figure 3-7. Bile acid pool composition in C57Bl/6J, CD-1, and 129/J mice. Bile acid composition of gallbladder bile was determined by the HPLC-ELSD method described in this chapter and the ratios were determined using the peak areas of the corresponding bile acids. Values represent means ± SE of data from 6 mice from each strain. Differences among groups of mouse strains were assessed for statistical significance by using single-factor analysis of variance (ANOVA). This was followed by least significant difference test as a post hoc test. B group is significantly different ($P < 0.01$) from A groups.

3.4 Discussion

The analysis of bile acids in biological samples has always presented technical difficulties because of their complex and diverse structures. GC-MS has been used for many years but the method is expensive, not commonly available and tedious, as it requires extraction, purification, hydrolysis of conjugates, and preparation of volatile derivatives (Dionne *et al.*, 1994). Over the past fifteen years liquid chromatography-mass

spectrometry has been used for qualitative analysis of bile acids (Setchell and Vestal, 1989; Eckers *et al.*, 1990; Libert *et al.*, 1991; Gelpi, 1995) and has only recently been used successfully to quantitate bile acids (Perwaiz *et al.*, 2001). While these methods of bile acid measurement are accurate, they can be costly, time consuming, and complex in their upkeep and usage. HPLC methods of bile acid analysis have existed for over 30 years. While these methods are more facile due to the complex nature of bile acids, universal separation of bile acids has been difficult. The method described in this chapter is a culmination of HPLC methods of the past that have utilized acetonitrile or ionic strength buffers (Heuman, 1989). Previous HPLC methods have relied on the detection of bile acids by UV absorption and have always been problematic. In the last 10 years, ELSD has been shown to be a superior method of bile acid detection (Criado *et al.*, 2003). Published HPLC-ELSD methods require a gradient method, which introduces a loss of sensitivity from improper atomization of the mobile phase, or are not able to analyze all naturally occurring bile acids. The advantage to the HPLC-ELSD system described in this chapter is that it is both isocratic and can separate all bile acids tested in the least amount of time of any published HPLC method so far. This developed method represents a simple, cost-effective and convenient approach to the routine analysis of bile acids.

Analysis can be done directly on biological samples using the system presented in this chapter, which has previously proven difficult (Criado *et al.*, 2003). Since other lipids present in bile did not interfere with the bile acid analysis (Figure 3-5), all that was required to prepare samples was a dilution and centrifugation to eliminate particles. In GC-MS or other available bile acid assays, purification is usually required. In experiments utilizing the method described here, samples taken from mice were analyzed within minutes of animal sacrifice in a quick and facile manner. Similarly, any biofluid could be analyzed by this method. The only potential problem is that while impurities may not interfere with bile acid signal, they could overload

the unretained peak or collect on the column without periodical cleaning. Lipid extraction samples from homogenized tissue or feces have also been successfully analyzed for bile acids by this method without interference from other sterol or lipophylic molecules present. The sensitivity of the procedure is in the sub-nanomole range with distinct separation of the major species of bile acids that are found in both human and rodent bile.

The HPLC-ELSD method described in this chapter represents a sensitive and versatile procedure for the detection and quantitation of bile acids. Before this method was developed, the only means this laboratory had to measure bile acids was through a commercial enzymatic assay or TLC. While the enzymatic assay has proved useful in the past for simple experimentation, in order to characterize and determine the function of the bile acid-binding ILBP, a more dependable and sensitive method of bile acid detection became a necessity for this thesis. A reliable means to measure and identify bile acid species is now established in the laboratory. In later chapters, this method is utilized to measure and identify acidic sterols in gallbladder bile, bile duct secretions, feces, and from organ lipid extractions. Furthermore, the method described in this chapter also enables a more sophisticated *in vitro* binding study of ILBP that was previously impossible in the laboratory. I performed a bile acid-binding competition assay to determine the bile acid binding preference of recombinant ILBP using the HPLC-ELSD system described in this chapter. This study, the preferential binding of ILBP and other characterizations of this protein, are the subject of Chapter 4.

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Chapter 4

The Relative Ligand Binding Preference of Murine Ileal Lipid-Binding Protein

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4.1 Introduction

ILBP is expressed abundantly in the ileum (Gong *et al.*, 1994), the major site of active bile acid absorption in the gut. Initial analysis of recombinant rat, porcine, and human ILBP suggested that ILBP binds bile acids and fatty acids (Sacchettini *et al.*, 1990; Miller and Cistola, 1993; Fujita *et al.*, 1995). However, a subsequent study showed that ILBP bound a bile acid with a higher affinity than a fatty acid (Gong *et al.*, 1994) suggesting that the primary function of ILBP is the binding of bile acid.

Although it has long been hypothesized that ILBP facilitates the intracellular shuttling of bile acids in the enterocyte of the ileum, the binding specificity of ILBP toward individual species of fatty acids or bile acids remains unknown. However, the binding selectivity towards the species of both types of lipids has been ascertained in L-FABP, the intracellular lipid-binding protein with the closest evolutionary relationship with ILBP (Hanhoff *et al.*, 2002; Schaap *et al.*, 2002). Studies have determined that L-FABP prefers to bind fatty acids with longer chain lengths and decreasing double bond number (Richieri *et al.*, 2000) and bile acids that are more hydrophobic (Thumser and Wilton, 1996). Though the portal region of the protein appears to be involved in the regulation of ligand exchange, the binding specificity of L-FABP is determined by the hydrophobicity of the ligands present and their ability to associate with the interior hydrophobic pocket of the protein (Thompson *et al.*, 1999). By comparison, the cellular retinol-binding protein II (CRBP-II) preferentially binds retinol and retinoic acid derivatives over fatty acids (Li *et al.*, 1987). Among the different fatty acids tested, however, it was found that CRBP-II interacts best with stearic acid (Richieri *et al.*, 2000). Therefore, in contrast to L-FABP, the preferential binding of CRBP-II toward different fatty acid species demonstrates more binding selectivity. In this chapter the binding selectivity of murine ILBP (mILBP) toward both fatty

acids and bile acids was probed. To this end, mILBP was expressed in *Escherichia coli* (*E. coli*) and the lipid binding activity of the purified recombinant protein was analyzed *in vitro*.

4.2 Methods

4.2.1 Expression and purification of recombinant murine ILBP

Dr. Qianqian Li constructed a pmILBP expression vector while working in Dr. L. Agellon's laboratory. The cDNA containing the open reading frame encoding mILBP from C57BL6/J mice was amplified using the 5' primer (5'-CACCATGGCTTCAGTGGCA-3' containing an *NcoI* site, the ATG start codon and the next 4 amino acid) and the 3' primer (5'-CCAAGCTTGGTAGCTCAAGC-3' containing the stop codon TGA and a *HindIII* site). The amplified sequence was verified by DNA sequencing. The primary structure of encoded mILBP is identical to the previously published sequence (Crossman *et al.*, 1994). The 405 bp PCR product was inserted into the pKK233-2 plasmid vector at the *NcoI* and *HindIII* sites. The resulting plasmid, named pmILBP, was introduced into *E. coli* BL21 *Codon plus* (Stratagene, La Jolla, CA, USA) and was used to direct the inducible expression of recombinant mILBP. Production of recombinant mILBP was induced by the addition of isopropylthio-β-D-galactoside (IPTG) to a final concentration of 0.45 μM once an OD_{600} of 0.450 had been attained. Cells were incubated for an additional 2 h and then harvested by centrifugation (10,000 x g for 10 min at 4°C). The bacterial pellet was suspended in a solution of 20 mM Tris (pH 8.0), 0.05% NaN_3, 0.1 mM DTT, *Complete*™ protease inhibitor cocktail (Roche Diagnostics, Laval, QC, Canada), and then lysed in a French press under 70 kg/cm^2. The resulting lysate was

centrifuged at 15,000 x g for 20 min at 4°C to remove insoluble debris. Solid ammonium sulfate was slowly added to the supernatant while stirring at 4°C until 60% saturation had been reached. The precipitated proteins were sedimented by centrifugation at 15,000 x g for 20 min at 4°C. The supernatant was passed through three 5 ml *Econo-Pac*™ methyl HIC cartridges (Biorad, Hercules, CA, USA) connected in series on an FPLC (Amersham Pharmacia Biotech Inc.) at a flow rate of 2 ml/min. Protein bound to the column was then eluted using 20 mM Tris-HCl (pH 8.0) buffer and fractions were analyzed by SDS-PAGE (Laemmli, 1970). The fractions containing the 14 kDa protein were pooled and passed through three 5 ml *Econo-Pac*™ High Q cartridges (Biorad) connected in series on the FPLC at a flow rate of 2 ml/min. The flow-through was collected and dialyzed against a buffer containing 20 mM potassium phosphate (pH 7.4), 0.05% NaN_3, 0.1 mM DTT and 100 mM NaCl. The dialyzed protein solution was concentrated to 9-10 mg/ml using an Amicon stirred cell (Millipore, Bedford, MA, USA) and then applied to a Hi-Prep 26-60 Sephacryl S100 column (Amersham Pharmacia Biotech Inc.) equilibrated with the same potassium phosphate buffer. Chromatography was done at a flow rate of 0.5 ml/min and fractions were analyzed by SDS-PAGE with silver staining. Fractions containing only the 14 kDa protein were pooled. The purified protein was concentrated to 1 mg/ml in a stirred cell. Delipidation of the protein was accomplished by passage through a hydroxyalkoxypropyl dextran type VI column (Sigma-Aldrich) at 37°C as previously described (Glatz and Veerkamp, 1983). The purified recombinant mILBP was used as an antigen to generate an antiserum in rabbits according to standard procedures.

4.2.2 Sedimentation equilibrium experimental procedures

Analytical ultracentrifugation experiments were performed by L. Hicks in cooperation with Dr. C. Kay. The determination of protein concentration for these analyses was calculated by fringe count analysis using a Beckman XLI Analytical ultracentrifuge and double-sector capillary synthetic boundary sample cells (Beckman Coulter Inc., Mississauga, ON, Canada). The samples were dialyzed for 48 h prior to analysis. The absorbance of the samples was then measured using a Perkin Elmer Lambda spectrophotometer and 0.2 cm pathlength cuvettes (Perkin Elmer, Wellesley, MA, USA). Samples of 150 μl were then loaded into one sector of the sample cell and 400 μl of dialyzate samples were loaded into the other sector. The run was performed at 8,000 rpm, and scans were taken when fringes could be resolved across the boundary region between the protein solution and the solvent. The number of fringes produced was then measured and the protein concentration was calculated using an average refractive increment of 3.31 fringes/mg/ml (Babul and Stellwagen, 1969).

Sedimentation equilibrium experiments were carried out at 20°C in a Beckman XLI Analytical Ultracentrifuge (Beckman Coulter Inc.) using interference optics following the standard procedures. Aliquots of 110 μl of sample solution (1 mg/ml) were loaded into six-sector sample cells, allowing three concentrations of sample to be run simultaneously. Analyses were performed at three speeds and each speed was maintained until there was no significant difference in scans taken 2 h apart to ensure that equilibrium was achieved. The sedimentation equilibrium data was evaluated using a non-linear least-squares curve-fitting algorithm (Johnson *et al.*, 1981). The partial specific volume of the proteins was calculated from the amino acid compositions (Cohn and Edsall, 1943).

4.2.3 MALDI-TOF mass spectrometry

Matrix assisted laser desorption ionization time of flight (MALDI-TOF) mass spectrometry was performed by the Institute for Biomolecular Design (University of Alberta, Edmonton, Alberta). The mass of mILBP was analyzed by MALDI-TOF mass spectrometry (Applied Biosystems Voyager 6064, Foster city, CA, USA) equipped with delayed extraction. The calibration matrix used was sinapinic acid and the linear standardization was calibrated using myosin.

4.2.4 CD spectrum determination

Circular dichroism (CD) analysis was performed by R. Luty in cooperation with Dr. C. Kay. CD spectra were recorded at 20°C in a Jasco J720 spectropolarimeter using the Jasco Hardware Manager ver1.10.00 and the J-700 Control Driver version 1.20.00 with a quartz cell with a path length of 0.02 cm (Jasco Inc., Easton, MD, USA). The protein concentration was 0.005 M, as determined by fringe count analysis, in a 20 mM potassium phosphate (pH 7.4) and 100 mM NaCl buffer. Spectral analysis was done in the presence or absence of 50% trifluoroethanol. Molar ellipticity values were calculated using a value of 113.175 Da for the mean residue weight calculated from the amino acid sequence of mILBP. Analysis of the secondary structure of the protein was performed using the CONTIN (Provencher and Glockner, 1981) procedure over the range of 250 to 192 nm using the Jasco Standard Analysis version 1.20.00 program.

4.2.5 Radiolabeled bile acid binding assays by gel filtration

Delipidated recombinant mILBP (27 nmol) and 170 nmol of [3H]-taurocholic acid (47,300 dpm/nmol) or [14C]-cholic acid (104,000 dpm/nmol) were incubated together for 1 h at 37°C in buffer composed of 20 mM potassium phosphate (pH 7.4), 0.05% NaN3, and 100 mM NaCl. The mixture was then applied to a Sephadex G25 16/40 column equilibrated with the same buffer at 4°C. An aliquot of each fraction was examined for radioactivity and protein concentration. This analysis was repeated with lysozyme, a protein with no known lipid binding activity.

4.2.6 Fatty acid analysis

The protein-fatty acid complex was obtained by incubating the fatty acid/ethanol solution and the mILBP solution (27 nmol in 20 mM potassium phosphate pH 7.4, 0.05% NaN_3, 100 mM NaCl) separately at 37°C for 1 h and then mixed together with vigorous stirring. The protein-fatty acid mixture was applied to a G25 column in the same manner described above to remove the remaining unbound fatty acids. The bound fatty acids were phase extracted from the protein using a modified Bligh method (Bligh and Dyer, 1959), and derivatized by methylation for GC analysis. The fatty acid methyl esters were separated and quantitated using an Agilent 6890 series GC system (Agilent Technologies, Palo Alto, CA, USA) equipped with an Agilent HP-23 column (cis/trans FAME 30 m x 0.25 mm x 0.25 μm) and a flame ionization detector operated by Priscilla Gao. Nitrogen was used as the carrier gas with pressure of 1.5 kg/cm^2, the detector temperature was 300°C, and the injector temperature was set at 220°C. The oven was initially set to 100°C and increased to 140°C at a rate of 10°C/min, followed by an

increase to 240°C at a rate of 3°C/min. Peak area data were analyzed using Chemstation software (Agilent Technologies). Detector linearity and calibration factors were determined using commercial standards (Sigma-Aldrich). Prior to lipid extraction, a heptadecanoic acid (C17:0) internal reference was added. Fatty acids were identified based upon retention times and quantitated based on peak areas relative to the peak area for C17:0.

4.2.7 Bile acid analysis

The protein-bile acid complex was obtained by incubating delipidated mILBP solution (20 mM potassium phosphate pH 7.4, 0.05% NaN3, and 100 mM NaCl) with a 5-fold molar excess of the bile acids at 37°C for 1 h. The unbound ligand was removed by size exclusion gel filtration chromatography as described above. The fractions containing protein were pooled and the bound bile acids were extracted using C18/14 Spe-ed SPE cartridges (Applied Separations, Allentown, PA, USA). The bile acids were eluted with methanol, dried down under nitrogen gas, and redissolved in the desired volume of methanol for chromatographic analysis. Bile acid recognition and mass analysis was performed with an HPLC coupled to an evaporative light scattering detector (ELSD) utilizing the method described in the previous chapter (Torchia et al., 2001). Peaks were identified based on their retention times compared to authentic bile acid standards and an internal standard of nor-DCA was included before bile acid extraction described in the sample preparation to quantitate bile acid mass.

4.3 Results

4.3.1 Expression and purification of recombinant mILBP

The pmILBP expression vector encoded a protein of approximately 14.3 kDa in *E. coli*. This recombinant protein was not detected in cells transformed with the empty vector. The purified protein (Figure 4-1, lane 3) was used to generate rabbit antiserum. Immunoblot analysis using the immune serum demonstrated an immunoreactive 14.3 kDa protein only in lysates transformed with the pmILBP expression vector (Figure 4-2A). This antiserum also detected a 14.3 kDa protein present in only the ileum of the murine small intestine (Figure 4-2B), where ILBP is known to be localized (Agellon and Torchia, 2000), thus confirming the identity of the recombinant protein as mILBP. Further, mass analysis by sedimentation equilibrium (14,188 ± 5%) and MALDI-TOF (14,354) of delipidated recombinant mILBP revealed a molecular mass consistent with the natural mILBP (Crossman *et al.*, 1994).

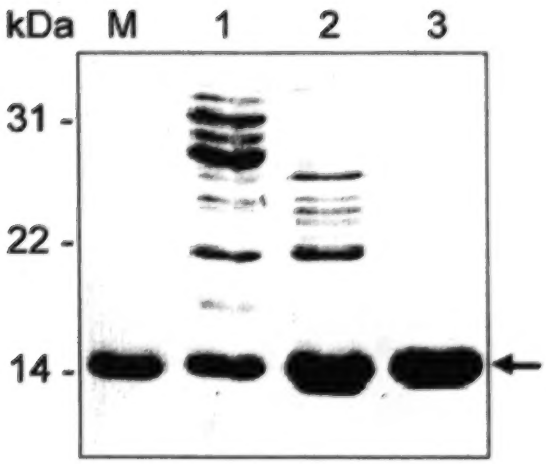

Figure 4-1. Expression and purification of a protein from E. coli transformed with pmILBP. Shown is a representative 18% SDS-PAGE of *E. coli* protein samples (20 μg each lane). Lane M, lysozyme marker; lane 1, sample of the protein after chromatography on an HIC column; lane 2, flow-through after application to an anion exchange column; lane 3, the purified protein after Sephacryl S100 gel filtration.

4.3.2 Binding of fatty acids to mILBP

Analysis by sedimentation equilibrium of the undelipidated form of mILBP revealed a global fit molecular weight of 14,677 ± 5%, which was slightly higher than delipidated mILBP, suggesting that a ligand may be bound to the protein. In a previous report, bacterial fatty acids were associated with recombinant porcine ILBP purified from *E. coli* (Lucke *et al.*, 1996). Gas chromatographic analysis of the lipids extracted from recombinant mILBP confirmed that fatty acids were associated with mILBP (Figure 4-3). The delipidation process removed more than 95% of the total lipids. Stearic and palmitic acids (at 37.6% and 34.5%, respectively) comprised the major fraction of the total fatty acids bound by mILBP. The remaining fatty acids consisted of C18:1 (at 13.1%), C16:1 (at 5.0%), C14:0

(at 4.9%), and a mixture of C20:0, C12:0, and others (at 5.0%). These results demonstrate that mILBP can bind fatty acids that are normally found in bacteria.

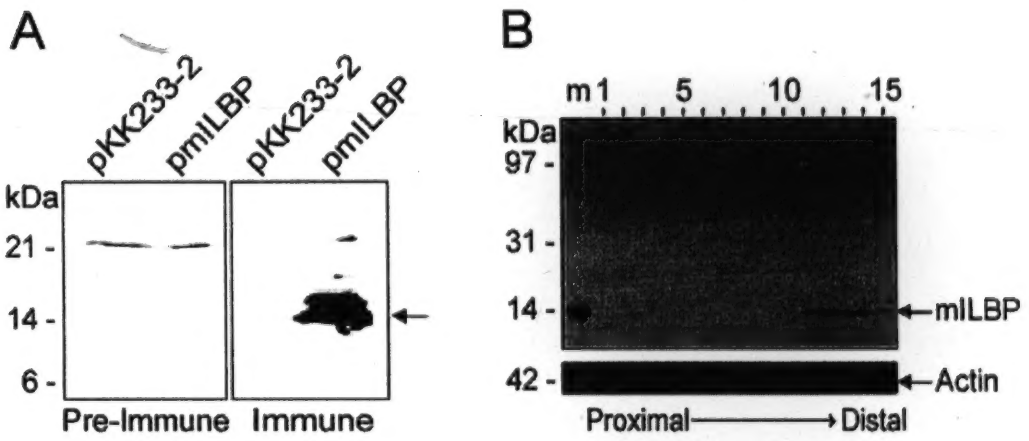

Figure 4-2. Identification of the purified protein as mILBP by immunoblot analysis. (A) The antiserum raised against the purified protein detects a band at approximately 14.3 kDa in *E. coli* lysates (20 μg) transformed with pmILBP, but not from the lysates (20 μg) of *E. coli* transformed with the empty vector pKK233-2. The preimmune serum did not detect the 14.3 kDa protein in either of the *E. coli* lysates (20 μg each). (B) The antiserum raised against the recombinant protein detected a protein in the distal portion of the small intestine. Lane m, recombinant mILBP; lanes 1-15, lysates (20 μg total protein in each lane) of mouse small intestine that was sectioned into fifteen equal segments from the proximal to the distal ends. Actin was detected in each of the lanes containing intestinal lysates utilizing an anti-actin antibody.

To test if mILBP exhibits a preference for a certain species of fatty acids, equal amounts of different fatty acid species were presented simultaneously to the protein. The lipids that bound to mILBP or lysozyme, a well characterized protein of similar molecular mass that is also known to not

have lipid-binding activity, were analyzed and quantitated by gas chromatography. Delipidated mILBP bound five times more fatty acid mass than lysozyme (Table 4-1). Similar to most intracellular lipid binding proteins including L-FABP, mILBP prefers fatty acid ligands with increasing chain length and saturation (Richieri *et al.*, 1994; Richieri *et al.*, 2000). These results demonstrate that fatty acids can be bound by mILBP *in vitro* and that this interaction is influenced by fatty acid hydrophobicity.

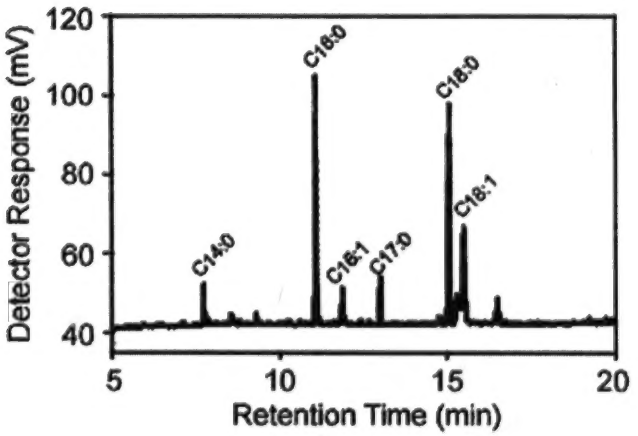

Figure 4-3. Endogenous fatty acids bound to recombinant mILBP. A representative chromatogram of lipid extractions from undelipidated mILBP analyzed by GC coupled with a FID. The fatty acids shown are myristic acid (C14:0), palmitic acid (C16:0), palmitoleic acid (C16:1), stearic acid (C18:0), and *cis*-vaccenic acid (C18:1). Heptadecanoic acid (C17:0) was added prior to solvent lipid extraction as a reference.

Table 4-1. Fatty acid ligand competition binding assay for mILBP and lysozyme.

	Fatty acids bound to mILBP (% of total bound by mILBP)						
Protein	C14:0	C16:0	C18:0	C18:1	C18:2	C18:3	C20:4
Lysozyme	2.1 ± 0.9	1.8 ± 0.6	2.8 ± 0.6	3.1 ± 1.3	3.0 ± 0.9	4.7 ± 0.5	2.1 ± 0.8
mILBP	5.3 ± 1.5	33.3±7.4	37.9±3.2	14.7±0.5	7.7 ± 2.6	N.D.	N.D.

Data (mean ± SD) are expressed as a percentage of the total mean amount of fatty acids bound by mILBP (n=6). Delipidated mILBP or lysozyme was incubated with an equal 5-fold molar excess of each fatty acid species indicated. Unbound lipids were separated from bound lipids by Sephadex G25 gel filtration and the protein fractions were collected and pooled. The lipid extractions of the pooled fractions were methylated and analyzed by GC coupled with an FID. 100% represents 23 nmol of total fatty acids bound to mILBP. The limit of detection of the analytical procedure is 0.40 pmol. N.D., not detectable.

4.3.3 Binding of bile acids to mILBP

The bile acid binding activity of mILBP was analyzed *in vitro*. Sedimentation equilibrium mass estimation analysis of mILBP in the presence of a 5-fold molar excess of TCA revealed an increase in protein mass. The association of bile acids with mILBP was further analyzed by gel filtration chromatography. Aliquots of lysozyme or mILBP protein solutions were incubated with excess radiolabeled TCA prior to chromatographic analysis. As shown in Figure 4-4, a larger proportion of radiolabeled TCA associated with mILBP compared to lysozyme. A small amount of TCA was found to co-elute with lysozyme, but this is likely due to non-specific binding. In the absence of mILBP, no radiolabeled TCA was detected in the corresponding fractions. Similar results were obtained when radiolabeled cholic acid was used as the bile acid in the analysis. Quantitation of the

amount of TCA bound by mILBP revealed a stoichiometry of two bile acids bound per molecule of mILBP. These data demonstrate the ability of recombinant mILBP to bind bile acids *in vitro* and in the absence of other proteins or membranes. Furthermore, this work suggests that mILBP may obtain ligand through a diffusion-mediated transfer process similar to L-FABP instead of a dependent membrane interaction ligand donation process exhibited by I-FABP (Thumser and Storch, 2000). In support of this, it has recently been determined that ILBP has a relatively more solvent-accessible protein cavity (Lucke *et al.*, 2002), which may facilitate free ligand exchange.

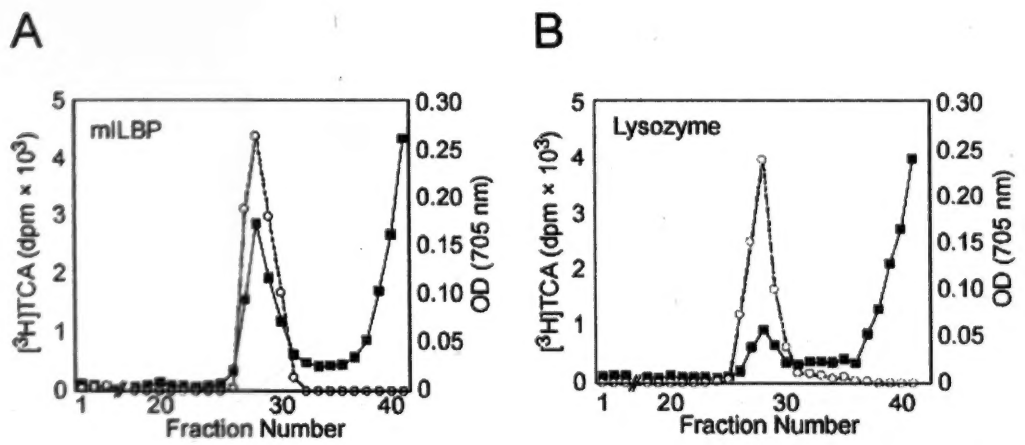

Figure 4-4. Murine ILBP binds taurocholic acid. Recombinant mILBP (A) and lysozyme (B) were incubated with radiolabeled [^{3}H]-taurocholic acid. Unbound bile acids were separated from bound bile acids by Sephadex G25 gel filtration. Fractions were collected and assayed for radioactivity (•) and protein concentration (○).

In order to determine if mILBP can discriminate different species of bile acids, equal amounts of different unconjugated or conjugated bile acid species were presented to the protein in 5-fold molar excess in two separate sets of experiments. mILBP was recovered from the reaction mixture and the bound bile acids were analyzed by HPLC-ELSD. The analysis revealed that, among the unconjugated bile acids tested, the rank order of binding is DCA = β-MCA > CDCA >> CA (Table 4-2). No binding was detected for LCA. The binding preference of mILBP to unconjugated bile acids species appears to be dependent on both the level of hydroxylation of the steroid moiety of a bile acid and also the position of these hydroxyl groups.

To ascertain the importance of bile acid hydroxylation at the 6 position more work was done using murocholic acid, which resembles LCA with a 6β-hydroxyl group. The bile acid competition assay was repeated using murocholic acid, DCA, CDCA, CA, and LCA. The results of this second experiment revealed that mILBP bound murocholic acid comparably with the amount that β-MCA bound in the first experiment (ratio 0.97). This suggested that hydroxylation at the 6 position improves the relative affinity of a bile acid to mILBP.

Among the conjugated bile acids, mILBP preferred to bind GCDCA >> TCDCA = β-TMCA >> TCA > GCA (Table 4-2). Thus mILBP can bind both unconjugated and conjugated bile acids *in vitro*. Moreover, mILBP exhibits preferential binding to some bile acid species according to the specific configuration of hydroxylation of the steroid moiety.

Table 4-2. Preferential bile acid binding of mILBP for unconjugated or conjugated bile acids.

	Bile acids	% of total bound
A.	Unconjugated bile acids	
	β-muricholic acid	33.2 ± 4.5
	Cholic acid	3.3 ± 0.2
	Chenodeoxycholic acid	26.2 ± 2.8
	Deoxycholic acid	37.3 ± 4.1
	Lithocholic acid	N.D.
	Total	100%
B.	*Conjugated bile acids*	
	Tauro-β-muricholic acid	21.5 ± 2.6
	Taurocholic acid	0.8 ± 0.8
	Glycocholic acid	0.2 ± 0.2
	Taurochenodeoxycholic acid	24.3 ± 2.8
	Glycochenodeoxycholic acid	53.2 ± 6.4
	Total	100%

Data (mean ± SD) are expressed as a percentage of total unconjugated bile acids (in A) or conjugated bile acids (in B) bound to mILBP (n=8 for each group). Delipidated mILBP or lysozyme was incubated with either conjugated or unconjugated bile acids, which contained an equal 5-fold molar excess of each species indicated. Unbound bile acids were separated from bound bile acids by Sephadex G25 gel filtration and the protein fractions were collected and pooled. Bile acids were extracted from the pooled fractions with a C18/14 cartridge and analyzed by HPLC coupled with an ELSD. 100% represents 48 nmol and 36 nmol for A and B, respectively. The limit of detection of the analytical method is 0.08 nmol. N.D., not detectable.

The previous analysis tested unconjugated and conjugated bile acids separately. To determine if conjugation influenced the relative affinity of the bile acid to mILBP, bile acids from both classes were presented simultaneously to the protein. The chromatogram in Figure 4-5 reveals the bile acids that were bound preferentially by mILBP. In general, conjugation of the steroid moiety to glycine or taurine improved binding of the bile acid to mILBP. This was true even for relatively weak binders such as cholic acid, although the improvement in relative affinity did not increase to a level that was comparable to the other unconjugated bile acids. Interestingly, TCA in excess amounts can saturate mILBP as shown in Figure 4-4, even though it is a weak binder in comparison to other bile acid species.

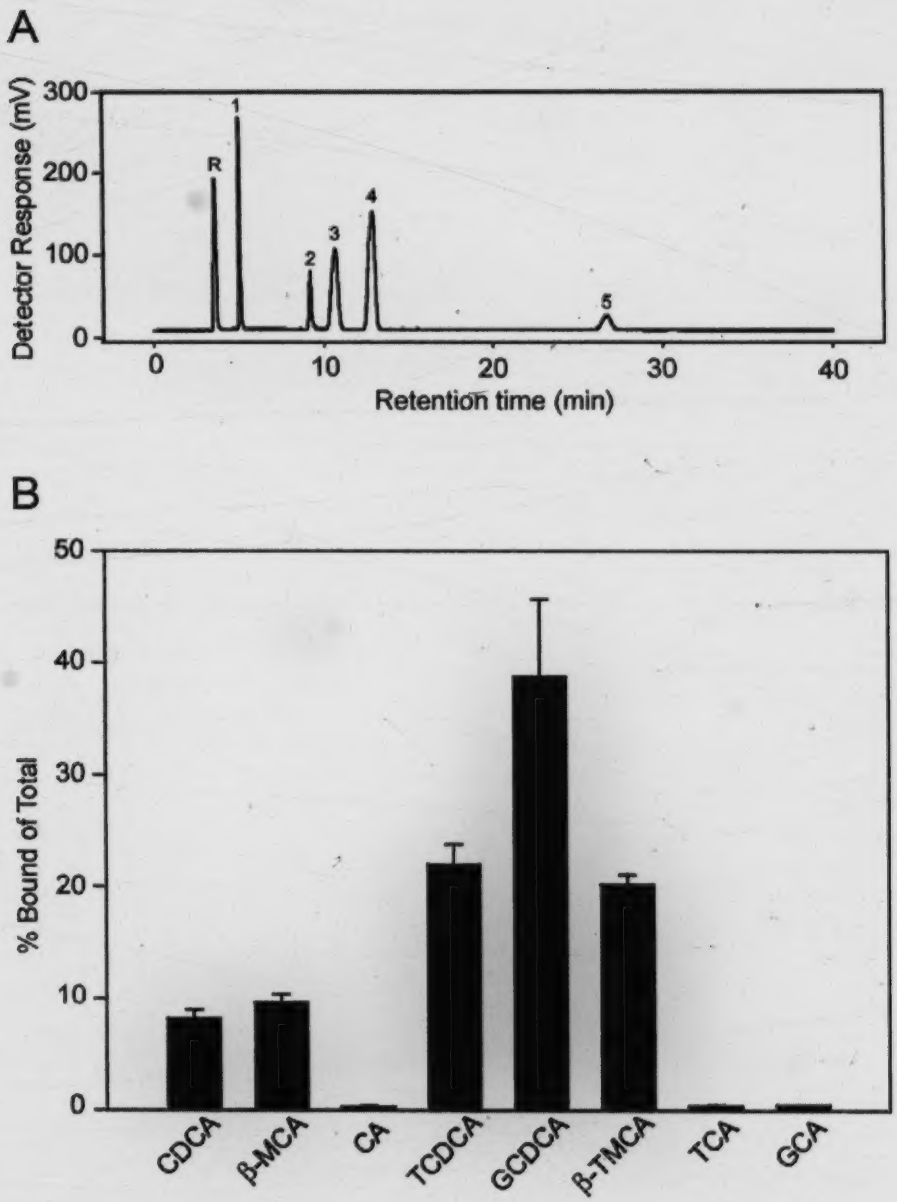

Figure 4-5. Bile acid competition binding assay. A 5-fold molar excess of each of the bile acids indicated was incubated with recombinant mILBP. Unbound bile acids were removed by gel filtration and the bile acid population bound by mILBP was identified and quantitated by HPLC-ELSD. (A) The bile acids assayed and their retention times are shown in a representative chromatogram. (B) Quantitation anlysis results expressed as a percentage of the total bound (mean ± SD, n = 8).

4.3.4 Influence of ligand on mILBP secondary structure

The secondary structure of mILBP in ligand bound and unbound states were determined by far-UV circular dichroism measurements. The spectra of the delipidated form of mILBP with and without the addition of a 5-fold molar excess of TCA are shown in Figure 4-6. Analysis of the data collected over the range 192 to 250 nm by the CONTIN procedure (Provencher and Glockner, 1981) provided estimates of secondary structure (Table 4-3). The data from the analysis showed a statistically significant increase of β-sheet character (from 0.54 ± 0.022 to 0.65 ± 0.031) when the delipidated protein was in the presence of TCA. However, the difference was not deemed quite enough to be distinct due to the error inherent in the CD method. Therefore, the increase in β-sheet character is seen as slight. The secondary structure of the undelipidated form of the protein initially purified from *E. coli*, containing fatty acids, also tended to have increased β-sheet character (0.57 ± 0.032), but not to the same extent as when TCA was present. These data imply that fatty acids may not be as tightly bound by mILBP compared to bile acids. Taken together, these findings are suggestive of a slight increase in structural order when recombinant mILBP is in the bound state. This is in agreement with NMR studies comparing the bound and unbound states of ILBP (Lucke *et al.*, 2002; Kurz *et al.*, 2003).

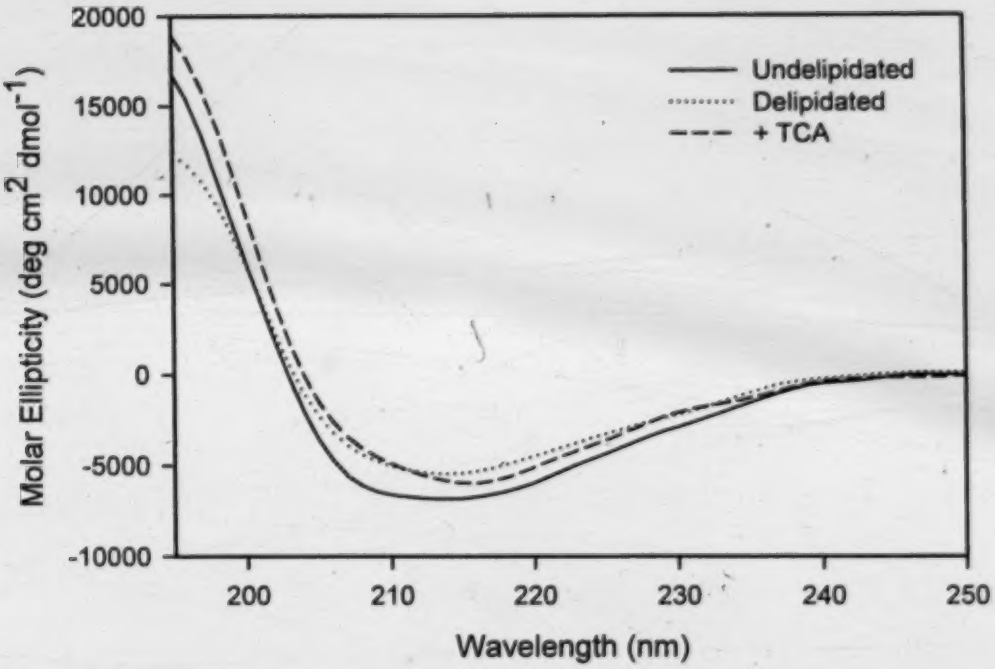

Figure 4-6. Analysis of the ligand-bound mILBP by circular dichroism. Far ultraviolet circular dichroic spectra of undelipidated mILBP, delipidated mILBP, and delipidated mILBP treated with excess taurocholic acid (+ TCA). The spectra were recorded at 20 °C in 20 mM potassium phosphate buffer (pH 7.4) and 100 mM NaCl, with a path length of 0.02 cm. Each spectrum represents an average of 8 scans.

Table 4-3. Secondary structure estimates of the different forms of mILBP listed calculated from the CD spectra using the CONTIN procedure.

mILBP	α-Helix	β-Sheet	β-Turn	Remainder
Undelipidated	0.10 ± 0.02	0.57 ± 0.03	0.27 ± 0.03	0.05 ± 0.04
Delipidated	0.08 ± 0.01	0.54 ± 0.02	0.26 ± 0.02	0.12 ± 0.01
Delipidated + TCA	0.06 ± 0.02	0.65 ± 0.03	0.21 ± 0.01	0.08 ± 0.03

4.4 Discussion

Although recent NMR studies have revealed the structure of ILBP in a ligand bound state (Lucke *et al.*, 1996; Lucke *et al.*, 1999; Lucke *et al.*, 2000; Kramer *et al.*, 2001), relatively little is known about the lipid binding activity of this protein. The initial characterization of ILBP when it was first cloned demonstrated that this protein might bind both bile acids and fatty acids. The data presented in this chapter support this initial assessment of ILBP ligand binding. Subsequent ligand binding studies found that oleic acid could not displace 7,7-azo-taurocholic acid that is bound to ILBP (Gong *et al.*, 1994) suggesting that ILBP binds bile acids more strongly than fatty acids. In order to bind a bulky and rigid class of lipids such as bile acids, studies have shown ILBP to be remarkably flexible (Lucke *et al.*, 1996; Lucke *et al.*, 1999) and to have one of the largest binding pockets found in the intracellular lipid-binding protein family (Lucke *et al.*, 1996; Lucke *et al.*, 2000). One of the first studies probing ILBP bile acid binding reported a 1:1 ligand-protein stoichiometric ratio (Sacchettini *et al.*, 1990). A subsequent study utilizing isothermal titration calorimetry to estimate binding constants suggested that 2 moles of bile acids may be binding to one mole of rat ILBP (Miller and Cistola, 1993). The data from this chapter are in agreement with this last observation. While structural NMR studies describing models of human and porcine ILBP in a ligand-bound state show only one bile acid molecule bound to a protein, a more recent NMR binding study suggests a model with two binding sites with weak intrinsic affinities that display positive binding cooperativity (Tochtrop *et al.*, 2002; Tochtrop *et al.*, 2003). The authors of this last study further explain that earlier NMR structure determinations may have missed the second binding site due to temperature-dependent NMR exchange broadening. It is worth noting that ILBP has two openings on the surface of the protein, similar to L-FABP, which binds two fatty acid

molecules at once (Thompson *et al.*, 1999). However, it is not known whether the second opening plays a role in bile acid binding. It is surprising that ILBP can bind two bile acid molecules given the bulky and rigid nature of the steroid framework.

While the purpose of ILBP bile acid binding may remain a mystery, binding energetic studies reveal how ILBP may operate within the enterocyte. Compared to other intracellular lipid-binding proteins, ILBP binds bile acids in a more permissive fashion and fatty acids very weakly (Zimmerman *et al.*, 2001; Hanhoff *et al.*, 2002; Tochtrop *et al.*, 2002). Therefore, while ILBP may facilitate the intracellular shuttling of bile acids, which display much higher monomer solubility compared with fatty acids and retinoids, this protein also allows a number of these amphiphilic lipids to exist in an unbound and accessible state at lower concentrations (Tochtrop *et al.*, 2003). At higher bile acid intracellular concentrations, the cooperative binding described above would allow ILBP to sequester a larger population of the bile acids in order to perhaps protect the enterocyte from bile acid-induced apoptosis or cytotoxicity. Further physical analysis may help elucidate the role of ILBP in the shuttling of bile acids in the cytoplasm.

The limited structural studies described in this chapter agree with what is currently known about intracellular lipid-binding proteins. The results of our CD experiments indicated that mILBP is predominantly β-pleated sheet with negligible amounts of α-helix (Table 4-3), consistent with the data from known crystal structures of other intracellular lipid binding-proteins (e.g. the rat intestinal fatty acid binding protein consists of 14% α-helix, 81% β-fold, and 5% remainder) (Jones *et al.*, 1988; Sacchettini *et al.*, 1989; Cowan *et al.*, 1993; Banaszak *et al.*, 1994; Mei *et al.*, 1997). Further, mILBP secondary structure measured in this study agrees with NMR data for the porcine orthologue of this protein (Lucke *et al.*, 1996). The slight increase in β-sheet character observed in the lipid-bound form of this highly structured protein is

difficult to quantitate by CD analysis, but may nevertheless be physiologically important.

The purpose of the present chapter was to determine if mILBP has a preference for specific species within each of the two lipid classes. Since ILBP binds fatty acids weakly (Miller and Cistola, 1993) and not at all with any fluorescent lipid probe tested (Hanhoff *et al.*, 2002; Tochtrop *et al.*, 2003), the binding kinetics of ILBP for various species of fatty acids is notoriously difficult to determine and remains unknown. The binding preference of mILBP towards different fatty acid species was determined in the present chapter using a competition binding assay. Increased chain length and saturation improved the ability of mILBP to bind fatty acids. Thus, the hydrophobicity of the fatty acids tested appears to be a major determining factor dictating its interaction with mILBP. It is interesting to note that intracellular lipid-binding proteins with different binding affinities for fatty acids all have a similar fatty acid binding preference hierarchy (Richieri *et al.*, 1994; Schroeder *et al.*, 1995; Richieri *et al.*, 1998; Richieri *et al.*, 2000) with a few exceptions. Some intracellular fatty acid binding proteins have developed specialized binding for eicosanoids or very long chain fatty acids with varying degrees of unsaturation not tested here (Hanhoff *et al.*, 2002). Fatty acids are likely attracted to the hydrophobic binding pocket within the β-clam structure of these proteins that is protected from the aqueous environment.

By contrast, we have determined that bile acid binding by mILBP is independent of bile acid hydrophobicity. Rather, two distinct factors appear to influence the selectivity of binding. The first factor is the hydroxylation state of the steroid moiety. Of the bile acids hydroxylated at the 3, 7, or 12 positions, a doubly hydroxylated steroid moiety seemed to be preferred. This was surmised after observing that CA (a trihydroxy bile acid) was weakly bound by mILBP compared to either DCA or CDCA (dihydroxy bile acids) and that LCA (a monohydroxy bile acid) was not bound at all. One noted

exception to this observation was the trihydroxy bile acid β-MCA (hydroxylated at the 3, 6 and 7 positions) which bound mILBP as well as CDCA and DCA. These data indicate that the hydroxyl group at the sixth position improves the binding affinity of a bile acid for mILBP. Furthermore, since murocholic acid (3α,6β-dihydroxy-5β-cholanic acid) bound equivalently to β-MCA, hydroxylation of the position 7 was not necessary for potent binding by mILBP. Mouse bile contains 6β-hydroxy bile acids (Torchia *et al.*, 2001), thus the preference of mILBP to this type of bile acid is not unexpected. One of the proposed functions of ILBP is the delivery of bile acid ligands to FXR to facilitate gene regulation. Both DCA and CDCA are stronger activators of FXR than CA (Makishima *et al.*, 1999). Since mILBP prefers to bind DCA and CDCA over CA, this bile acid binding protein could be involved with this putative mechanism for regulation of bile acid-related genes. Additionally, stronger binding of DCA and CDCA compared to CA by mILBP may be advantageous since CA displays reduced toxicity compared to DCA and CDCA (Garewal *et al.*, 1996; Pritchard and Watson, 1996; Sodeman *et al.*, 2000). Thus, despite the fact that the mouse enterohepatic circulation contains a large amount of CA, the bile acid-binding preference of mILBP may have been strategically developed to aid in the metabolic processing of bile acids by enterocytes.

The second factor is conjugation of the side chain to taurine or glycine. In general, conjugation further improved the interaction of mILBP with bile acids. This was evident even in a weakly bound bile acid such as CA. However, conjugation of CA was not sufficient to raise the binding of this bile acid to the level of the unconjugated dihydroxy bile acids CDCA and DCA. Conjugated bile acids predominate in the enterohepatic circulation. Uptake of these bile acids in the ileum requires active transport by ASBT (Agellon and Torchia, 2000). Not surprisingly, conjugated bile acids are the preferred substrate of this transporter. ASBT has been suggested to interact

with ILBP possibly loading bile acids with ILBP (Kramer *et al.,* 1995; Kramer *et al.,* 1997). However, it is apparent that this interaction is not necessary for ILBP to bind bile acids *in vitro*. Common substrate preferences between ILBP and ASBT may indicate cooperation of function between these two proteins.

Recently, there have been two other studies which examined ILBP bile acid binding. The first utilized tryptophan fluorescence to monitor protein binding since conventional methods of binding affinity determination are difficult to apply to ILBP (Zimmerman *et al.,* 2001). Their study of human ILBP binding also shows that conjugation improves the interaction of bile acids with ILBP. However, similar to an earlier study using rat ILBP (Gong *et al.,* 1994), taurine conjugation improved binding to a greater degree than glycine. Furthermore, Zimmerman et al. report a DCA>CA>CDCA binding hierarchy for bile acids for human ILBP instead of the DCA>CDCA>CA reported here. Indeed, the predominant bile acid species in the enterohepatic circulation and the primary structure of ILBP are not identical among different animals. Thus, the binding preference of ILBP may be specifically tailored to bile acids that are present in a given animal. It is interesting to note, however, that for both humans and mouse, hydroxylation at the 7 position seems to decrease binding while hydroxylation at the 12 position improves the binding of a potential ligand. Additionally, the data in this chapter suggest that hydroxylation at the 6 position is an important binding factor in the murine model. Since the human enterohepatic circulation contains no bile acids hydroxylated at the 6 position, it is not surprising that the response to the hydroxylation pattern of bile acids would be different from ILBP in either species.

. A second recent study examining bile acid binding of human ILBP utilized isothermal calorimetry titration to determine binding affinities and, taking into account the second binding site, binding cooperativity (Tochtrop *et*

al., 2003). They report that the relative affinities between bile acid species are not very different but that the binding cooperativity did differ. The present study does not differentiate between these two factors but rather would represent an amalgamation of the two. Indeed, the study performed by Tochtrop et al. demonstrates that bile acid mixtures seem to reflect averages of the values between the corresponding individual binding isotherms. Therefore, the mixing of bile acids results in direct competition for the binding sites of ILBP without many secondary effects. The *in vitro* bile acid competition assay performed in this chapter should reflect the *in vivo* bile acid binding preference of ILBP. Furthermore, the mixing of bile acid species may be more physiologically relevant than measuring the binding capabilities of one bile acid at a time. However, a major weakness of this approach is that since no binding affinity calculations can be made, comparison of binding abilities to other proteins is impossible. Further, if cooperative binding is different for each bile acid species as reported by the Tochtrop group, then the binding preferences of mILBP may be somewhat variable in response to bile acid concentration. Another weakness to this approach is that competition and displacement between two rival ligands are crucial. If one ligand has a binding affinity of more than 3 orders of magnitude different than another, it will appear to never displace and therefore not bind to the protein. For example, LCA may bind weakly to ILBP but appears to not bind at all in Table 4-2. Another example of this is that CA conjugated and unconjugated bile acids may appear to weakly interact with ILBP (Table 4-2, Figure 4-5) but it would be more accurate to describe it as weak only in comparison to DCA. This is the chief reason why it was impossible to make direct comparisons between fatty acid and bile acid binding. Nevertheless, the competition binding assay accomplishes the goal of this chapter to establish the binding hierarchy preference of murine ILBP in the presence of an abundance of mixed bile acids.

In summary, the binding of fatty acids to mILBP was most influenced by fatty acid hydrophobicity. For bile acids, mILBP exhibited binding selectivity which was determined primarily by hydroxylation of the steroid moiety while relative binding affinity was influenced by amino acid conjugation. In chapter 5, the possible functions of ILBP will be discussed as a mouse model deficient in ILBP is examined.

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Chapter 5

Ablation of the Ileal Lipid-Binding Protein Reduces Intestinal Bile Acids

5.1 Introduction

As indicated in Chapter 1, intestinal reclamation of bile acids is necessary to maintain an enterohepatic cycling of bile acids involving the liver, the gallbladder, and the small intestine. Bile acid transporter activities in these organs are vital to maintain this enterohepatic circulation. Indeed, mutations in the human *ASBT* gene (Oelkers *et al.*, 1997) or the disruption of murine *ASBT* gene (Dawson *et al.*, 2003) greatly reduces bile acid absorption in the distal gut which, due to a decrease in the bile acid pool, in turn leads to the hindrance of intestinal uptake of lipophilic macronutrients. While the role of many transporters in the enterohepatic circulation of bile acids may be straightforwardly defined, the contribution of ILBP to bile acid trafficking remains unclear. Ever since ILBP was identified as a soluble enterocytic protein with the putative ability to bind bile acids (Sacchettini *et al.*, 1990; Miller and Cistola, 1993; Fujita *et al.*, 1995), this protein was immediately recognized as a potential transporter of bile acids for intracellular trafficking. As I have observed in previous chapters, ILBP is a relatively well characterized protein still in search of a function. Several characteristics, such as the ileal-specific intestinal expression and the binding affinity, suggest that ILBP may be crucial for bile acid trafficking and, in turn, the enterohepatic cycling of these molecules.

Intracellular lipid-binding proteins such as ILBP have been postulated to facilitate intracellular trafficking of lipid ligands. Indeed, mice lacking adipocyte lipid-binding protein (AP2), another member of the intracellular lipid-binding protein family, retain the ability to shuttle lipids in adipocytes but show a decrease in basal lipolysis, a decrease in lipid efflux, and an increase in intracellular non-esterified fatty acids in these fat cells (Coe *et al.*, 1999). Lipid processing in the adipocyte is hampered but not lost in mice lacking AP2. A concomitant increase in expression of other intracellular lipid-binding

proteins present in adipocytes may account for this observation. Despite this increase in expression, the total amount of intracellular lipid-binding proteins is greatly reduced in fat cells of *AP2* null mice. Thus, while members of the intracellular lipid-binding proteins may differ in lipid-binding preference, a redundancy may have evolved to ease intracellular lipid trafficking. Likewise, ablation of the *L-FABP* gene results in a marked decrease in hepatic fatty acid utilization and turnover efficiency, despite the increase in expression of other intracellular lipid-binding proteins (Martin *et al.,* 2003a, b; Newberry *et al.,* 2003). Similarly, ILBP may be implicated in efficient directional trafficking of bile acids in enterocytes involving a lipid exchange mechanism which has yet to be determined.

The loss of ILBP may result in a great reduction of bile acid enterohepatic cycling. However, there are two additional features that ILBP possesses which may make it unique among proteins of the same family. The first is that ILBP is the only cytosolic protein expressed in enterocytes whose primary function is to bind bile acids (Lin *et al.,* 1990; Lin *et al.,* 1991). The other two intracellular lipid binding proteins, I-FABP and L-FABP, show either no affinity for these large, rigid lipid molecules or have binding affinities several order of magnitudes lower than ILBP. Therefore, unlike hepatocytes or adipocytes that have at least 2 redundant intracellular lipid-binding proteins to bind fatty acids, in the enterocyte it appears that no other cytosolic protein could compensate for the loss of ILBP. The second point is that several studies have demonstrated an interaction between ASBT and ILBP (Kramer *et al.,* 1993; Kramer *et al.,* 1995; Kramer *et al.,* 1997; Kramer *et al.,* 1998). While ASBT activity does not require ILBP, ILBP may modulate ASBT function or assist directly in the transport of bile acids. No other intracellular lipid-binding protein has been shown to associate with intermembrane transporters. Therefore, the contribution of ILBP to the conservation of bile acids in the enterohepatic circulation may be two-fold: to facilitate the translocation of bile acids from the apical to the basolateral

membrane of an enterocyte and to facilitate transporters of bile acids at these membranes. In the present Chapter, potential roles that ILBP may play in bile acid metabolism are examined. Therefore, to determine if ILBP is crucial for the preservation of bile acids in the enterohepatic circulation, the phenotype of mice lacking ILBP is examined.

5.2 Methods

5.2.1 Animals and diets

Chimeric *ILBP*$^{-/-}$ mice were generated in Dr. Agellon's lab by Enrique Torchia using standard techniques (Torchia *et al.*, 2001). Briefly, a targeting vector for the *ILBP* gene was constructed to ablate expression of *ILBP* by the deletion of exons 2 and 3 of the *ILBP* locus. Linearized vector DNA was introduced into embryonic stem cells by electroporation. Embryonic stem cells that underwent homologous recombination at the *ILBP* locus were enriched by positive-negative selection. Surviving cell lines were isolated, expanded, and screened for targeting events. Positive embryonic stem cell lines were karyotyped using standard techniques (Abbondanzo *et al.*, 1993; Nagy *et al.*, 1993; Stewart, 1993) to verify genetic integrity. Embryonic stem cell lines having a low number of aneuploid karyotypes were injected into C57BL/6J blastocysts and placed in the uteri of pseudopregnant females. Chimeras proving to be germ line competent were crossed with C57BL/6J mice to produce heterozygous offspring. The resulting progeny were interbred to produce the *ILBP*$^{-/-}$ mice and wild type *ILBP*$^{+/+}$ littermate control mice. The 129J genetic contribution was diluted by backcrossing into C57BL/6J ten times to produce *ILBP*$^{-/-}$ mice which could be considered to have a C57BL/6J strain genetic background.

ILBP-deficient mice were bred and screened for approximately 2 years for the purposes of experimentation described in this Chapter. Rodent chow (Purina Rodent chow #5001) and water were provided *ad libitum*. The animals were maintained in a temperature-controlled (25°C) facility on a 12 h light/dark reverse light cycle. Unless otherwise indicated, animals were deprived of food for 8 h prior to the completion of experiments. Mice were then sacrificed between 7 am and 11 am following gaseous anaesthesia. Blood was collected *via* heart puncture or tail bleed using EDTA-treated needles and syringes. Within 3 hours, plasma was separated from all blood samples by low-speed centrifugation and stored at 4°C. Bile was collected once the gallbladder had been removed, cleaned, and subjected to filtration-centrifugation and stored at -80°C. Internal organs were excised, weighed, and flash frozen in liquid nitrogen and stored at -80°C. Portions of the ilium and liver were preserved in 10% buffered formalin at necropsy and used for histological analysis. The experimental protocols for the use of laboratory mice were approved by the University of Alberta Health Sciences Animal Welfare Committee and were performed in accordance with the guidelines established by the Canadian Council on Animal Care.

5.2.2 Hepatic enzyme activity assays

Cyp7a1 and HMGCR activity assays were performed by Le Luong using microsomes that were prepared from frozen liver as described previously (Cheema *et al.*, 1997) following homogenization with a Polytron PT 10-35 (Kinematica AG, Switzerland). Cyp7a1 specific activity was measured by the conversion of [4-^{14}C]-cholesterol (Amersham Pharmacia) into [4-^{14}C]-7α-hydroxycholesterol using a modification of a previously described HPLC method (Chiang, 1991). Purified microsomes were assayed (100 µg total protein/assay) using a modification of a previously described

isotope incorporation assay (Martin *et al.*, 1993). In these assays, [4-^{14}C]-cholesterol (10 mCi/mmol; 13 nmol per assay) encapsulated in β-cyclodextrin (Sigma) was used as substrate. Sterols were separated by thin layer chromatography using a ethyl acetate-toluene (3:2 v:v) solvent system. The amount of 7α-hydroxycholesterol was quantitated by using a Fuji BAS1000 phosphorimaging plate and the Quantity One software package (Biorad). Activity is expressed as pmol of 7α-hydroxycholesterol produced per minute per mg of microsomal protein. The HMGCR activity (pmol/min·mg protein) was assayed in the microsomes (250 µg protein) by determining the conversion of [^{14}C]-3-hydroxy-3-methylglutaryl-CoA (10 mCi/mmol; 1 nmol per assay) to [^{14}C]-mevalonate using standard methods (Shapiro *et al.*, 1974). The amount of mevalonate was quantitated using the imaging plate as described above.

5.2.3 Bile duct cannulation for collection of bile

This procedure was accomplished with the help of Rick Havinga from the laboratory of Dr. Folkert Kuipers (Groningen Institute for Drug Studies, The Netherlands) and is described in detail elsewhere (Kuipers *et al.*, 1996). Briefly, mice fasted overnight were anaesthetized (20 ml/kg) with a cocktail prepared by mixing 2.5 ml ketamine/xylazine solution (10 mg/ml and 2 mg/ml, respectively), 0.3 ml diazepam (5 mg/ml), and 2.2 ml saline. During the 90 min bile collection period, animals were placed in a humidified incubator to ensure maintenance of body temperature. After ligation of the common bile duct close to the duodenum, the gallbladder was cannulated using silastic tubing (internal diameter, 0.5 mm; external diameter 0.94 mm) and a dissecting microscope. Bile was immediately collected in pre-weighed tubes in 30 minute intervals for a total period of 90 min. Bile production was determined gravimetrically, assuming a density of 1 g/ml for bile, and bile

was stored immediately at -20°C for later colorimetric assays (cholesterol, phosphatidylcholine, and bile acids) and HPLC analysis. After 90 min, blood was collected by cardiac puncture using EDTA-coated syringes and the liver was removed, weighed, frozen in liquid nitrogen, and stored at -80°C. Plasma was obtained by centrifugation at 4000 rpm for 10 min and stored at 4°C until HPLC analysis as detailed in Chapter 2.

5.2.4 Bile acid and neutral sterol analysis

Extraction of bile acids from small intestine was done as previously detailed (Hedenborg *et al.*, 1986). Briefly, the small intestines of mice were collected and snap frozen in liquid nitrogen. Frozen tissue was homogenized in 95% ethanol containing 0.1% ammonia. Homogenates were incubated at 80°C after addition of [24-^{14}C]-GCA (5 µCi per sample) as an internal standard. Resulting extracts were filtered and a fraction from each extract was dried down and resuspended in 1 ml of methanol. A 100 µl aliquot was dried down for scintillation counting. Concentration of total bile acids was determined using a diagnostic bile acid kit from Sigma-Aldrich (St. Louis, MO).

Analysis of bile acids and neutral sterols in feces was accomplished using a modified Malchow-Moller method (Malchow-Moller *et al.*, 1982). Briefly, mice from each group (6 *ILBP*$^{+/+}$ male, 6 *ILBP*$^{+/+}$ female, 6 *ILBP*$^{-/-}$ male, and 6 *ILBP*$^{-/-}$ female) were kept in metabolic cages and droppings were collected daily over a period of three days. Each daily sample was desiccated, weighed, and homogenized in 10 ml of acidified ethanol (10% acetic acid, 90% ethanol). The addition of [^{14}C]-CA (5 µCi per sample) and [^{14}C]-cholesterol (5 µCi per sample) served as an internal standards. The supernatant was poured off, dried down, and resuspended in 2 ml 1 M HCl and 2 ml of ethyl acetate. The top phase was then removed and the phase

separation was repeated 3 times before drying down all the ethyl acetate collected. The residue was then resuspended in 1 ml of methanol, measured for neutral sterols by using a diagnostic cholesterol kit from Sigma-Aldrich, and extraction efficiency was determined using a scintillation counter. A 500 µl aliquot was dried down and resuspended in a 100 mM Tris pH 7.4 buffer. Solvent phase extraction was then accomplished with 1 ml of petroleum ether. The bottom phase was collected and the concentrations of total bile acids were determined using a diagnostic bile acid kit from Sigma-Aldrich. Extraction efficiency for bile acids was determined using a scintillation counter. The amount of bile acids in each daily sample was calculated and the average over a three day period was used to represent the data from one mouse.

5.3 Results

5.3.1 General characterization of the ILBP-deficient mice

Standard targeted gene disruption technology was used to produce mice deficient in ILBP by Dr. Enrique Torchia as outlined in his thesis (Torchia, 2001). The *ILBP* gene is a single copy gene (Crossman *et al.*, 1994) and is composed of 4 exons distributed within a small region of genomic DNA (~ 6 kb). The strategy utilized to disrupt the *ILBP* gene was to delete exon 2 and 3 utilizing the known sequence of the murine *ILBP* gene (Crossman *et al.*, 1994) through homologous recombination in embryonic stem cells. Dr. Torchia's efforts resulted in 20 chimeras from which only 3 germline mice were obtained. The mutated *ILBP* allele was detected in the chimeras and F1 generation by DNA amplification and DNA blotting analysis. Similar tests conducted on the F2 generation confirmed that the *ILBP*

mutation had been transmitted. This showed that Dr. Torchia had created mice that carried a gene disruption in the *ILBP* locus. RNA blot analysis revealed that no translatable or truncated *ILBP* mRNA was detectable in mice homozygous for the *ILBP* mutation. Using the ILBP antiserum described in Chapter 4, I performed an immunoblot analysis of *ILBP*-disrupted mice. Figure 5-1 shows two representative immunoblots of small intestine sectional extracts from a wild type mouse (m25 +/+) and a $ILBP^{-/-}$ homozygous mouse (m28 -/-). While the expression of ILBP in $ILBP^{+/+}$ animals was restricted to the terminal small intestine, $ILBP^{-/-}$ animals did not express ILBP. These experiments show that the expression of the *ILBP* gene is functionally disrupted in *ILBP* null mice. The creation of $ILBP^{-/-}$ mice resulted in mice with a mixed genetic background (129J:C57BL/6J). To minimize genetic variability, these mice have since been backcrossed 10 times with C57BL/6J mice to dilute the contribution of the 129J genetic strain.

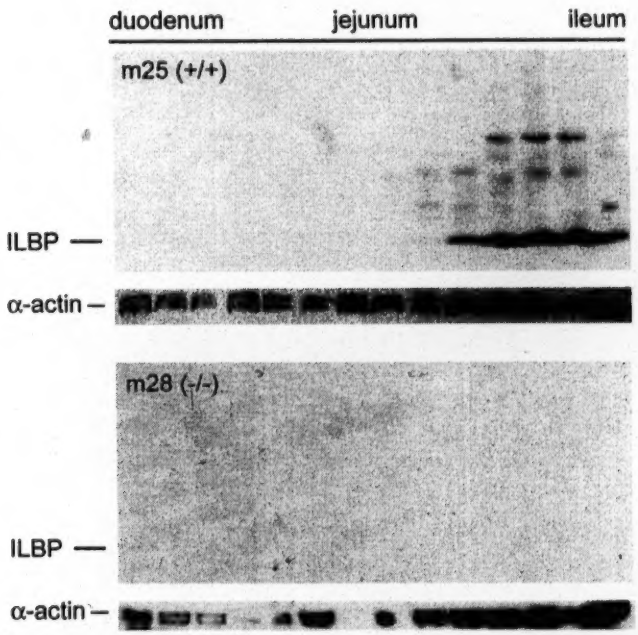

Figure 5-1. Detection of ILBP expression in protein extracts from mouse intestinal sections from *ILBP*$^{+/+}$ and *ILBP*$^{-/-}$ mice. Immunoblot analysis was performed using ILBP and α-actin antiserum. Blots shown are representative of several mice from F1, F2, and subsequent generations of *ILBP*$^{+/+}$ and *ILBP*$^{-/-}$ mice.

Ilbp null mice were viable and did not show any obvious abnormalities in morphology, behaviour, sex ratio, or fertility. Similar observations were made by Dr. Torchia when handling the first chimeras and the initial heterozygous crosses (Torchia, 2001). Observing the backcrosses and many subsequent generations over a period of 2 years has led me to agree with this initial assessment. Crossing heterozygous mice produced a Mendelian distribution of wild type and mutant genotypes. Furthermore, the fecundity and the sex ratio of the litters from the backcrossed *ILBP*$^{+/+}$ and *ILBP*$^{-/-}$ lines were similar. Body weight and postnatal development was of particular interest due to the phenotype that the *ILBP* null animals were

predicted to have. In humans, disrupting the return of bile acids to the liver in the enterohepatic circulation through bile acid malabsorption caused by a rare inherited *ASBT* mutation (Heubi *et al.*, 1982; Oelkers *et al.*, 1997) or ileal resection (Hofmann and Poley, 1972) results in a decreased efficiency in dietary lipid uptake and a concomitant decrease in body weight. If ILBP were required for conservation of bile acids in the enterohepatic circulation, then mice deficient in ILBP would not gain as much weight as their normal counterparts. As can be seen in Figure 5-2, there was no difference in post-natal growth or bodyweight of either male or female mice. Table 5-1 and Table 5-2 also show that body weight, daily food consumption, and daily fecal excretion (dry weight) were similar between the different genotypes for both genders of age-matched mice. Furthermore, the survival of *ILBP*$^{-/-}$ mice was indistinguishable from wild type littermates, unlike *Cyp7a1* null mice, which experienced a very high rate of postnatal mortality unless supplemented with exogenous fat-soluble vitamins and cholic acid (Ishibashi *et al.*, 1996; Schwarz *et al.*, 1996). However, this may not be surprising since mice lacking ASBT have been found to be resistant to any weight loss (no change in females and -20% in males) despite a decrease of ~80% in the bile acid pool and are able to thrive similar to their wild type littermates (Dawson *et al.*, 2003). *ILBP*$^{-/-}$ mice may equally be able to retain dietary lipid uptake efficiency despite a similar loss in the conservation of bile acids experienced by *ASBT* null mice. However, when Dr. Torchia compared the bile acid pools in *ILBP*$^{-/-}$ and *ILBP*$^{+/+}$ mice for both genders, he found that the sum of bile acids in gallbladder, liver, and small intestine remained unchanged despite the lack of ILBP (Torchia, 2001). Thus, from what we had seen from the outset and the data presented later in this Chapter, ILBP expression is not critical for conservation of bile acids in the enterohepatic circulation of mice.

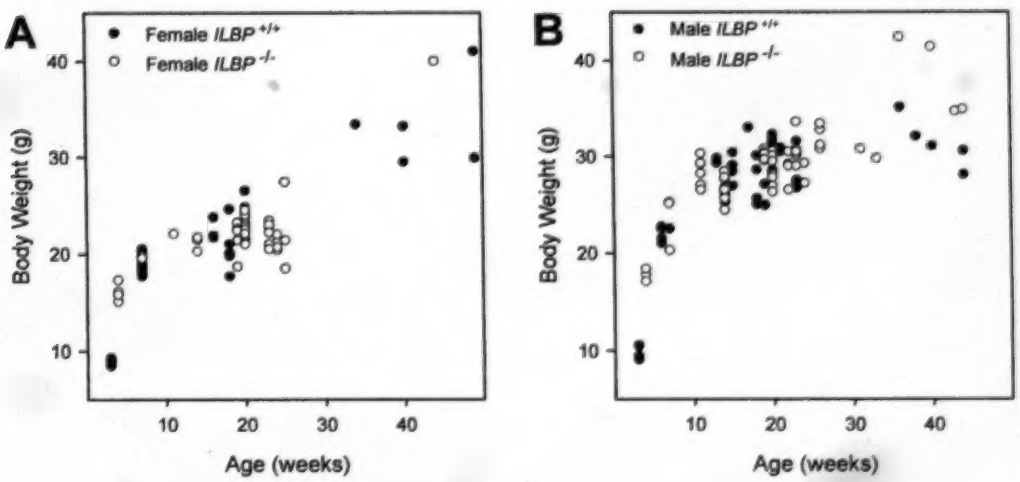

Figure 5-2. Growth of wild type and *ILBP*$^{-/-}$ mice. Offspring generated from heterozygous intercrosses after 10 generation backcrossing to C57BL/6J from the original chimeras were weighed. Each point represents a weight for a mouse only weighed once at one given age. Postnatal weight gain was indistinguishable between *ILBP*$^{-/-}$ and *ILBP*$^{+/+}$ mice for both genders.

Table 5-1. Organ weight, body weight, fecal output, and hepatic enzymatic activities of *ILBP*$^{+/+}$ and *ILBP*$^{-/-}$ female mice aged 18-25 weeks fed normal chow.

Parameter	*ILBP*$^{+/+}$ females	*n*	*ILBP*$^{-/-}$ females	*n*	*p*
Body weight (g)	22.4 ± 0.6	16	22.1 ± 0.3	32	ns*
Fecal excretion (dry g/d per 100g bw)	4.41 ± 0.56	18	4.59 ± 0.39	18	ns
Food consumption (g/d per 100g bw)	14.0 ± 1.2	18	15.2 ± 1.7	18	ns
Liver weight (g)	0.91 ± 0.03	10	0.92 ± 0.03	14	ns
Liver weight/body weight (%)	4.19 ± 0.15	14	4.26 ± 0.15	10	ns
Intestine weight (g)	1.07 ± 0.04	6	1.09 ± 0.06	6	ns
Intestine weight/body weight (%)	5.44 ± 0.18	6	5.16 ± 0.24	6	ns
Cyp7a1 activity	13.3 ± 3.2	9	14.1 ± 4.5	9	ns
Hepatic HMGCR activity	41.5 ± 9.1	9	43.8 ± 5.2	9	ns

Data are expressed as the mean ± SE. *n* indicates the number of mice analyzed for each parameter. Fecal excretion units refer to g of dry weight excreted per day per 100g of mouse body weight; Cyp7a1 activity is expressed as the amount of 7α-hydroxycholesterol converted from cholesterol in pmol/min·mg protein; HMGCR activity is expressed as the amount of mevalonate converted from 3-hydroxy-3-methylglutaryl-CoA in pmol/min·mg protein. Significance was determined using a Student's *t* test. * ns, not significant.

Table 5-2. Organ weight, body weight, fecal output, and hepatic enzymatic activities of *ILBP*$^{+/+}$ and *ILBP*$^{-/-}$ male mice aged 18-25 weeks fed normal chow.

Parameter	*ILBP*$^{+/+}$ males	*n*	*ILBP*$^{-/-}$ males	*n*	*p*
Body weight (g)	28.8 ± 0.4	25	29.4 ± 1.3	23	ns*
Fecal excretion (dry g/d per 100g bw)	3.92 ± 0.18	18	3.66 ± 0.13	18	ns
Food consumption (g/d per 100g bw)	15.3 ± 0.9	18	15.8 ± 1.5	18	ns
Liver weight (g)	1.23 ± 0.03	13	1.26 ± 0.04	11	ns
Liver weight/body weight (%)	4.49 ± 0.08	13	4.40 ± 0.11	11	ns
Intestine weight (g)	1.22 ± 0.08	6	1.07 ± 0.04	6	ns
Intestine weight/body weight (%)	4.53 ± 0.54	6	4.01 ± 0.38	6	ns
Cyp7a1 activity	7.4 ± 2.0	9	7.6 ± 1.3	9	ns
Hepatic HMGCR activity	44.6 ± 6.1	9	72.6 ± 8.3	9	0.027

Data are expressed as the mean ± SE. *n* indicates the number of mice analyzed for each parameter. Fecal excretion units refer to g of dry weight excreted per day per 100g of mouse body weight; Cyp7a1 activity is expressed as the amount of 7α-hydroxycholesterol converted from cholesterol in pmol/min·mg protein; HMGCR activity is expressed as the amount of mevalonate converted from 3-hydroxy-3-methylglutaryl-CoA in pmol/min·mg protein. Significance was determined using a Student's *t* test. * ns, not significant.

Given the toxic properties of bile acids, ILBP-deficiency may result in the failure to sequester bile acids and to damage enterocytes or indirectly lead to liver damage due to the mismanagement of absorbed bile acids. However, during necropsy no evidence of inflammation in the intestine or liver could be detected in *ILBP*$^{-/-}$ mice. These organs appeared indistinguishable from their wild type counterparts and exhibited a normal pink hue. Furthermore, fasted animals displayed the usual distension of the gallbladder with normal colored bile. Weighing the liver of the animals (Table 5-1 and Table 5-2) also revealed no evidence of hepatomegaly. Similarly, comparison in weight and length of the small intestine between the two genotypes revealed no evidence of inflammation or adaptation. Histological assessments of small intestine samples and liver samples were conducted by Dr. Richard Uweira. The intestine showed no morphological changes, no

unusual enterocytic hyperplasia, apoptosis, or necrosis. The liver showed no signs of fibrosis, inflammation, or hepatocytic vaculation. Only the wild type male group showed some small signs of hepatic inflammation, likely due to an unrelated cage illness. Therefore, ileal ILBP expression does not appear essential for the cytoxic protection of the enterocyte or to function as a buffer to the liver from the high concentration of bile acids associated with intestinal bile acid reclamation.

A loss of bile acid conservation efficiency in the ileum could be masked by increased bile acid synthesis in the liver. To examine this possibility, hepatic Cyp7a1 and HMGCR activity was determined in ILBP-deficient mice compared to the wild type littermates. The rate-limiting step in bile acid biosynthesis, Cyp7a1, showed no difference in activity for both genotypes in either gender (Table 5-1 and Table 5-2). Cholesterol biosynthesis, a precursor to bile acids, seems to have increased marginally for *ILBP*$^{-/-}$ male mice but not for females, as indicated by HMGCR activity. Therefore, no compensatory increase in bile acid biosynthesis has taken place. Furthermore, since bile acids exert feedback regulation on Cyp7a1 (Dueland *et al.*, 1993) and hepatic HMGCR (Duckworth *et al.*, 1991; Shefer *et al.*, 1992) activities, these data would further suggest that the intracellular levels of bile acid in the liver remain largely unchanged. While both Cyp7a1 and HMGCR activities are regulated by many other factors, chief among them cholesterol, the small increase in HMGCR activity in male *ILBP*$^{-/-}$ mice could be indicative of a large change in bile acid metabolism that is being masked by the compensatory upregulation of proteins similar in function to ILBP. A redundant expression of similar proteins could be hiding the extent of the phenotype. To examine this possibility, the abundance of mRNAs corresponding to several known genes are compared in *ILBP*$^{+/+}$ and *ILBP*$^{-/-}$ mice later in this Chapter. First, however, to understand the extent of the effects of ILBP-deficiency on lipid metabolism, specifically bile acid, hepatic bile secretion and lipid management in *ILBP* null mice was characterized.

5.3.2 Effects of ILBP-deficiency on bile formation and bile composition

Despite the fact the liver is the only site of bile acid biosynthesis and is the target for serum bile acids that come from intestinal bile acid reclamation, hepatic bile acid concentration remains quite low. Hepatocytic bile acids are immediately secreted basolaterally to bile duct tubules upon synthesis or apical absorption from circulation. Lower concentrations of bile acids and relatively higher concentrations of a diverse group of interfering neutral sterols present in the liver conspire to make direct measurements of the intracellular concentrations of bile acids difficult. However, liver secretion function can be ascertained through bile analysis. Bile formation is sensitive and responds to the levels of available lipids in the liver. Therefore, both gallbladder bile and biliary secretions were examined in *ILBP* null mice in order to assess the transhepatic bile acid flux. Inspection of bile from the gallbladders of the $ILBP^{+/+}$ and $ILBP^{-/-}$ mice showed no outward differences in golden colour or sac turgidity upon fasting. Male mice from both genotypes had approximately 100 mM bile acid concentration and 2 nmol of total bile acids in gallbladder bile (n = 10, per group). Similarly, female mice displayed no differences between genotypes with bile acid concentrations and total bile acids measurements of approximately 165 mM and 4 nmol, respectively (n = 10, per group). Bile is secreted from the liver through tubules, down to the common bile duct, and is collected in the gallbladder where some processing of the bile occurs, including concentrating bile acids (from ~30 mM to ~100 mM for males; ~45 mM to ~165 mM for females). To determine if bile formation had been affected in ILBP-deficient mice, biliary output was then measured through common bile duct cannulation. The bile can therefore be examined before processing as it is secreted from the liver.

Bile secretion for both genders and genotypes was similar (Figure 5-3A, B) and biliary output rates of bile acids (Figure 5-3C) were also unchanged. Since biliary secretion of phospholipids and cholesterol is tightly coupled to that of bile acids (Verkade *et al.*, 1995), the output rates of these biliary lipids were also determined. As shown in Figure 5-3D and Figure 5-3E, the output rates of cholesterol and phosphatidylcholine (PC), respectively, were also unchanged. Therefore, the bile formation process is normal and unaffected in these mice. The speciation of bile acids in the gallbladder was determined by HPLC as detailed in Chapter 3. Taurine conjugates of CA and β-MCA are the predominant bile acid species in mice (Schwarz *et al.*, 1996; Turley *et al.*, 1998). As can be seen in Table 5-3, in bile taken from the gallbladders of wild type and *ILBP*$^{-/-}$ mice, bile acid speciation and conjugation ratios appear normal for all mouse groups. HPLC bile acid analysis of the hepatic biliary secretion also reveals comparable bile acid speciation in both genotypes for either gender (Figure 5-3). Taken together, these data strongly indicate that the bile acid levels in the liver were not affected by ILBP-deficiency and that likely similar levels of bile acids are returning to the liver from ileal absorption for all mouse groups. Therefore, ILBP is not necessary for the conservation of bile acids in the enterohepatic circulation, as mentioned in section 5.3.1, nor does it seem part of a rate determining step in the return of bile acids to the liver. Since the speciation of bile acids in bile is also unchanged, ILBP does not appear to confer bile acid species absorption specificity.

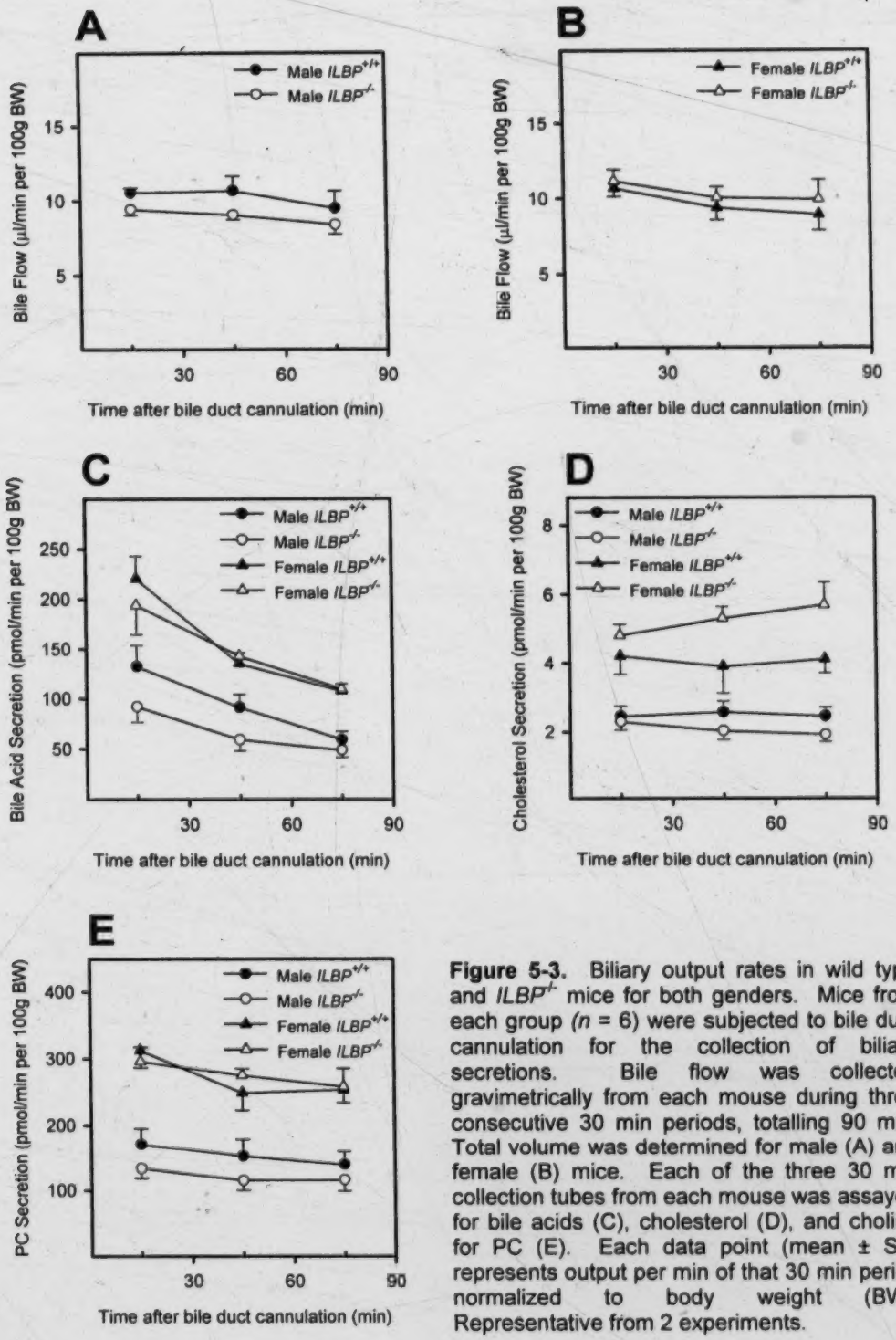

Figure 5-3. Biliary output rates in wild type and *ILBP*$^{-/-}$ mice for both genders. Mice from each group (n = 6) were subjected to bile duct cannulation for the collection of biliary secretions. Bile flow was collected gravimetrically from each mouse during three consecutive 30 min periods, totalling 90 min. Total volume was determined for male (A) and female (B) mice. Each of the three 30 min collection tubes from each mouse was assayed for bile acids (C), cholesterol (D), and choline for PC (E). Each data point (mean ± SE) represents output per min of that 30 min period normalized to body weight (BW). Representative from 2 experiments.

Table 5-3. Gallbladder bile acid concentration and composition (% total) in *ILBP*$^{+/+}$ and *ILBP*$^{-/-}$ mice.

	Male		Female	
Bile Acid (mM)	*ILBP*$^{+/+}$	*ILBP*$^{-/-}$	*ILBP*$^{+/+}$	*ILBP*$^{-/-}$
β-TMCA	55.2 ± 8.0	48.4 ± 5.8	81.0 ± 7.0	84.3 ± 9.3
TCA	46.9 ± 8.3	47.9 ± 8.6	80.1 ± 5.4	69.5 ± 4.3
TCDCA	0.9 ± 0.2	0.9 ± 0.1	1.0 ± 0.2	1.3 ± 0.2
GCA	0.7 ± 0.2	1.1 ± 0.3	3.0 ± 0.9	1.7 ± 0.3
GCDCA	0.3 ± 0.1	0.7 ± 0.2	0.9 ± 0.1	1.6 ± 0.2
β-MCA	0.4 ± 0.1	0.3 ± 0.1	1.3 ± 0.3	0.5 ± 0.1
CA	0.5 ± 0.1	1.1 ± 0.4	3.7 ± 1.0	1.1 ± 0.1
Total	106.6 ± 16.5	101.7 ± 14.7	174.1 ± 11.9	163.6 ± 14.3
Bile Acid (% Total)				
β-TMCA	53.1 ± 1.5	49.2 ± 2.6	46.4 ± 1.4	50.7 ± 1.5
TCA	42.7 ± 1.8	46.0 ± 2.2	46.4 ± 2.3	43.7 ± 1.7
TCDCA	0.7 ± 0.1	0.5 ± 0.1	0.6 ± 0.1	0.9 ± 0.1
GCA	1.0 ± 0.3	1.0 ± 0.2	1.7 ± 0.5	0.9 ± 0.1
GCDCA	0.3 ± 0.03	0.6 ± 0.1	0.5 ± 0.04	0.9 ± 0.1
β-MCA	0.6 ± 0.2	0.3 ± 0.1	0.7 ± 0.2	0.3 ± 0.04
CA	0.8 ± 0.2	1.0 ± 0.2	2.0 ± 0.5	0.6 ± 0.1

Values are expressed as means ± SE (n = 10) as the concentration of a bile acid specie in mM or a percentage of the total bile acids measured by HPLC. No significant differences were found between male or female groups

5.3.3 Effects of ILBP-deficiency on lipid homeostasis

Bile acids have been reported to affect both hepatic lipid secretions into bile and into blood (Verkade *et al.*, 1993; Repa *et al.*, 2000a; Miyake *et al.*, 2001). Therefore, another approach to examining the change in the

enterohepatic cycling of bile acids is to measure plasma lipid levels and to observe plasma lipid profiles. In the case of plasma triglyceride, previous *in vitro* experiments have shown an inverse relationship between bile acid concentration and hepatocyte VLDL-triglyceride secretion (Lin *et al.*, 1996a; Lin *et al.*, 1996b). Later, it was determined that feeding mice TCA results in a decrease of VLDL-triglycerides while cholestyramine-fed mice exhibited an increase in VLDL-triglycerides (Elzinga *et al.*, 2002). This is likely due to an inhibition of apolipoprotein B secretion by bile acids (Elzinga *et al.*, 2003). A more recent study has shown a close relationship between bile acid biosynthesis and triglyceride levels (Post *et al.*, 2004), likely due to the aforementioned regulation of apolipoprotein B secretion. For comparison purposes, three different mouse strains were fed normal chow alone or supplemented with either TCA or cholestyramine, a bile acid sequestrant. The effects of altering the bile acid pool on plasma triglyceride can be seen in Figure 5-4. As can be seen in each of the three plasma profiles (Figure 5-4 A-C), mice fed diets supplemented with TCA showed the predicted decrease in VLDL-triglycerides while cholestyramine-fed mice exhibited an increase. Indeed, the same trends can be seen in total plasma triglycerides (Figure 5-4 D); however, only 129J mice fed TCA had a significant decrease. If the bile acid pool of *I*$LBP^{-/-}$ mice were to be altered in comparison to its wild type counterparts, plasma triglyceride levels may emulate one of these scenarios.

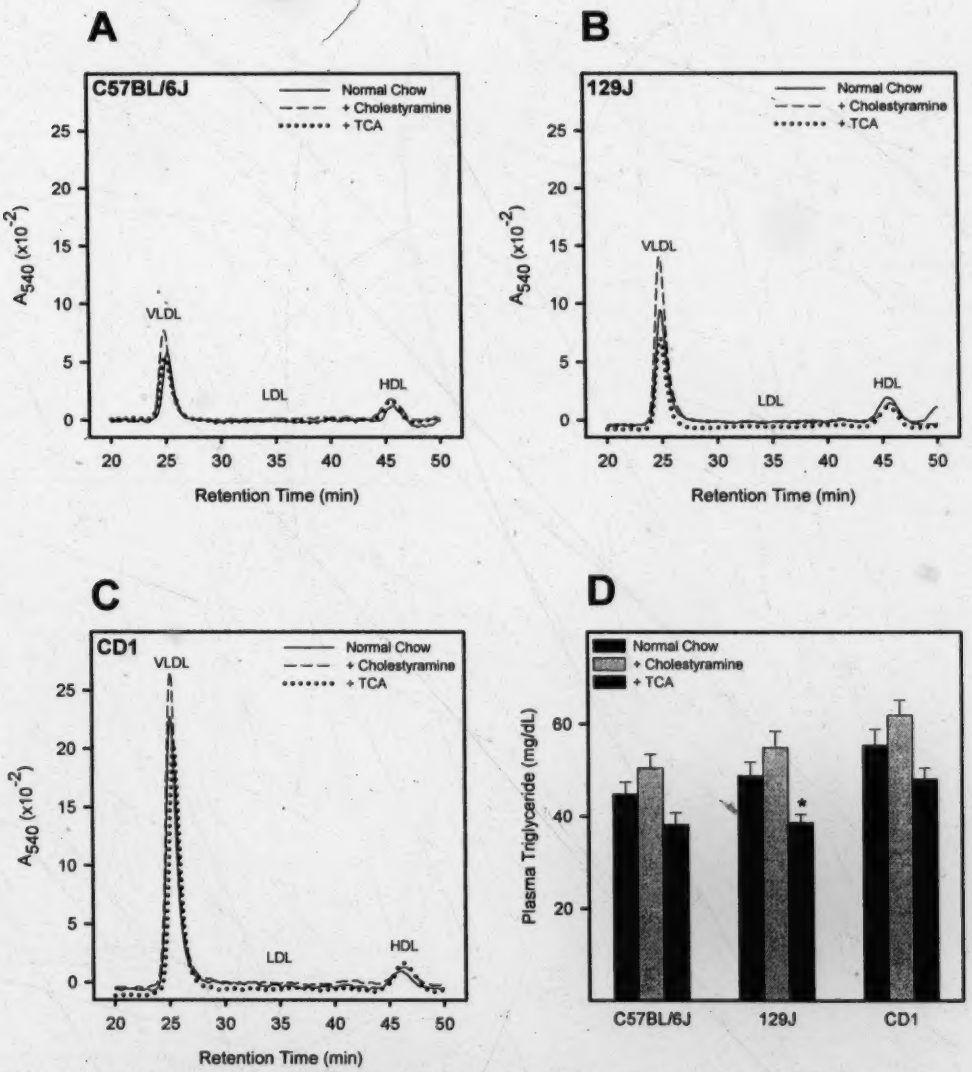

Figure 5-4. Plasma triglyceride analysis in three strains of female mice fed bile acids or bile acid sequestrants. Plasma samples from C57BL/6J (A), 129J (B), and CD1 (C) mice fed normal chow or normal chow supplemented with either 0.5% TCA or 2% cholestyramine for 2 weeks were analyzed for triglyceride content following FPLC gel filtration chromatograpghy. The data shown were produced by pooling equal amounts of plasma (n = 6) by strain-matched animals prior to analysis. Total triglyceride (D) was measured in fresh plasma from mice of the three strains listed (n = 6 for each group) by colorimetric assay. The data shown are mean ± *SE* and significance was determined using Student's t-test by comparing TCA-fed or cholestyramine-fed mice to mice fed normal chow. *, $P < 0.05$.

Recent findings have also shown that lipoprotein metabolism may be regulated by FXR. Given the triglyceride studies described above, it is not surprising that FXR-deficient mice display elevated plasma triglyceride levels when bile acid synthesis repression is inhibited (Sinal *et al.,* 2000). However, plasma cholesterol levels in these animals have also been shown to be elevated. This may be due to the role that FXR plays in HDL metabolism. Bile acid activated FXR is known to suppress apolipoprotein A-I transcription (Claudel *et al.,* 2002) and to mediate bile acid induction of phospholipid transfer protein expression (PLTP) (Urizar *et al.,* 2000), which plays a role in HDL remodelling (Jiang *et al.,* 1999). Moreover, previous studies using bile acid sequestrants demonstrated significant increases in HDL concentrations both in humans (Bard *et al.,* 1990; Hagen *et al.,* 1994) and animals (Staels *et al.,* 1996). Therefore, the effects of enhancing the bile acid pool, through TCA feeding, and disruption of the enterohepatic recirculation of bile acids, through cholestyramine feeding, were examined in three mouse strains. As can be seen in each of the plasma cholesterol profiles (Figure 5-5 A-C), cholestyramine-fed mice demonstrate elevated HDL-cholesterol presumably through the upregulation of apolipoprotein A-I. TCA-fed mice exhibited the corresponding decrease in HDL-cholesterol with a simultaneous increase in VLDL- and LDL-cholesterol. Figure 5-5D shows that total plasma cholesterol is affected by the dietary treatments. In murine plasma, HDL is the more abundant lipoprotein and, therefore, any increase in HDL could result in increased cholesterol mobilization. Indeed, a significant increase in total plasma cholesterol can be seen in all three strains fed cholestyramine. TCA feeding tended to decrease total plasma cholesterol levels (Figure 5-5D) and shift cholesterol from HDL to VLDL (Figure 5-5 A-C). Therefore, measuring plasma cholesterol levels in *I*$LBP^{-/-}$ mice may also prove useful in phenotype detection and reflect any changes in the bile acid pool of these mice.

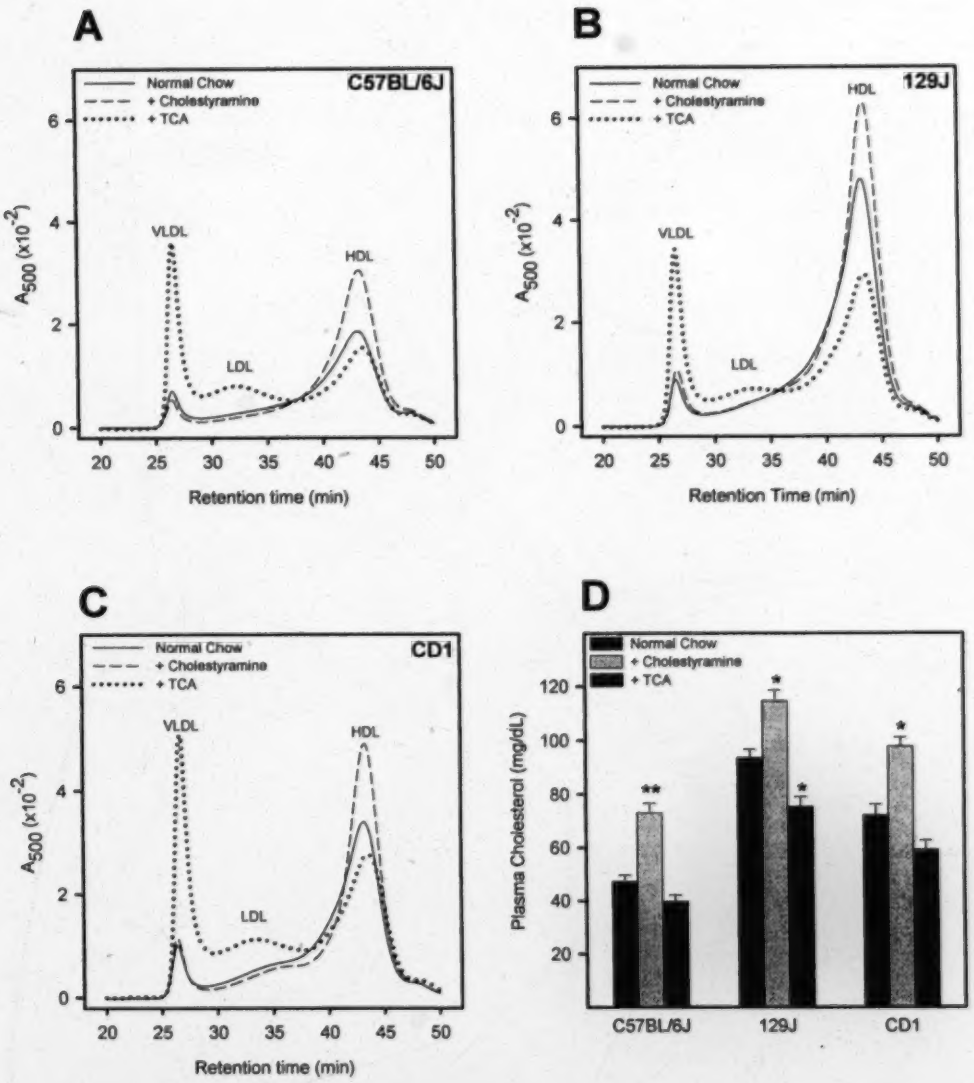

Figure 5-5. Plasma cholesterol analysis in three strains of female mice fed bile acids or bile acid sequestrants. Plasma samples from C57BL/6J (A), 129J (B), and CD1 (C) mice fed normal chow or normal chow supplemented with either 0.5% TCA or 2% cholestyramine for 2 weeks were analyzed for cholesterol content following FPLC gel filtration chromatograpghy. The data shown were produced by pooling equal amounts of plasma (n = 6) by strain-matched animals prior to analysis. Total cholesterol (D) was measured in fresh plasma from mice of the three strains listed (n = 6 for each group) by colorimetric assay. The data shown are mean ± *SE* and significance was determined using Student's t-test by comparing TCA-fed or cholestyramine-fed mice to mice fed normal chow. *, $P < 0.05$; **, $P < 0.01$.

The underlying hypothesis before undertaking this study was that the lack of ILBP would substantially decrease the bile acid pool. Given the predictable pattern of murine plasma lipid changes due to bile acid challenge established in Figure 5-4 and Figure 5-5, these findings should determine the validity of the hypothesis. If the bile acid pool has not been altered sufficiently then no difference in plasma lipids should be detected. Examination of the cholesterol lipoprotein profile in *ILBP*$^{-/-}$ male mice compared to control mice (Figure 5-6A) revealed that HDL-cholesterol and total cholesterol were lower suggesting an increase in bile acid pool size. However, no concomitant increase in VLDL-cholesterol consistent with an increased bile acid pool was detected. Analysis of female *ILBP*$^{-/-}$ mice showed no difference in plasma cholesterol compared to their wild type littermates. Based on the analysis described in section 5.3.2, there is likely no change in the amount of bile acids returning to the liver, in bile acid biosynthesis levels, or in liver lipoprotein secretion and remodelling. Therefore, the changes observed in *ILBP*$^{-/-}$ male mice (Figure 5-6A and B) likely have another cause, for instance, a change in hepatic cholesterol levels. In support of this, VLDL-triglyceride (Figure 5-6C) and total plasma triglyceride levels (Figure 5-6D) are reduced in both male and female mice to levels much lower than the dietary effect achieved in Figure 5-4. Therefore, it is improbable that the plasma lipids in *ILBP*$^{-/-}$ are responding to similar pressures seen in the supraphysiological dietary study conducted on the three strains of mice listed above.

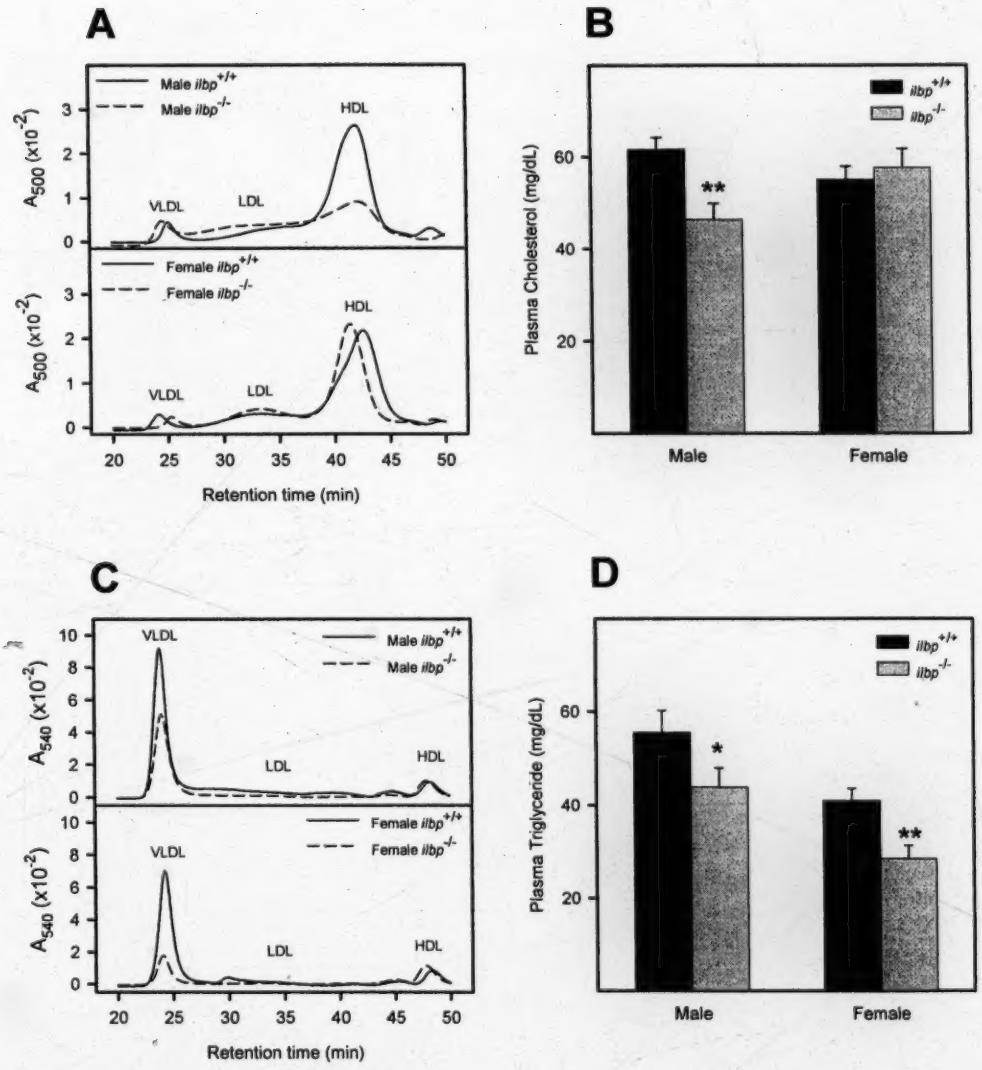

Figure 5-6. Plasma lipid analysis of *ILBP*$^{-/-}$ mice. Plasma cholesterol lipoprotein profiles (A) and total plasma cholesterol (B) measured from male (top) and female (bottom) mice using FPLC gel filtration and a colorimetric assay, respectively. Triglyceride lipoprotein profiles (C) and total triglyceride plasma concentrations (D) were similarly determined from the same plasma samples. The lipoprotein profiles shown were produced by pooling equal amounts of plasma (n = 6) by strain matched animals prior to analysis and are representative of two experiments. Histograms show mean ± *SE*. Statistical significance was assessed using *Student's t-test* by comparing *ILBP* null mice to wild type mice. *, $P < 0.05$; **, $P < 0.01$.

Given that ILBP is expressed at high levels in the ileocytes and not at all in hepatocytes, and that hepatic biliary function appears unperturbed in mice lacking ILBP, decreases in plasma cholesterol and triglyceride are more likely caused by intestinal loss of function than by altered liver-mediated lipoprotein metabolism. Specifically, intestinal absorption of lipid macronutrients may be less efficient in *ILBP*$^{-/-}$ mice. Figure 5-7 A and B shows the daily fecal excretion of bile acids and neutral sterols, respectively, in *ILBP*$^{+/+}$ and *ILBP*$^{-/-}$ mice of both genders. As expected from known gender differences, female bile acid excretion is higher than male. However, ablation of *ILBP* does not affect daily fecal bile acid excretion. These data suggest that loss of dietary lipid absorption efficiency is not due to a decrease in the bile acid pool resulting from poorer bile acid reabsorption. Furthermore, since bile acid loss must equal bile acid synthesis, there must also be no change in hepatic bile acid biosynthesis. This is supported by the observation in Table 5-1 and Table 5-2 that Cyp7a1 activity is unaltered in *ILBP* null mice. Figure 5-7B demonstrates that while wild type male neutral sterol daily fecal excretion may be somewhat lower than wild type females, male *ILBP*$^{-/-}$ mice have a higher neutral sterol excretion compared to male *ILBP*$^{+/+}$ mice. Similarly, ILBP status had no effect in female *ILBP*$^{-/-}$ mice. These data indicate that cholesterol absorption in the gut is likely decreased since it is being passed through to excretion in *ILBP*$^{-/-}$ males. This may explain the decrease in total plasma cholesterol and HDL-cholesterol in *ILBP*$^{-/-}$ males while *ILBP*$^{-/-}$ females exhibit no changes. At the same time, since *ILBP*$^{-/-}$ cholesterol biliary secretion is unaffected (Figure 5-3C), it may also explain the increased HMGCR activity in males while there is no change for females (Table 5-1 and Table 5-2). If dietary cholesterol absorption in *ILBP*$^{-/-}$ males were lessened, then an increase in cholesterol biosynthesis and in HMGCR activity would be desirable to maintain whole body cholesterol levels. This is especially important in the liver, the major source of

cholesterol biosynthesis. It must also be noted that the decrease in total and VLDL-triglycerides in both *ILBP*$^{-/-}$ genders may be due to less efficient fatty acid intestinal absorption. However, while a loss of dietary lipid absorption function may be etiologically involved with an *ILBP*$^{-/-}$ phenotype, it is not detrimental to ontogeny or adipogenesis as suggested by the body weight findings described in section 5.3.1.

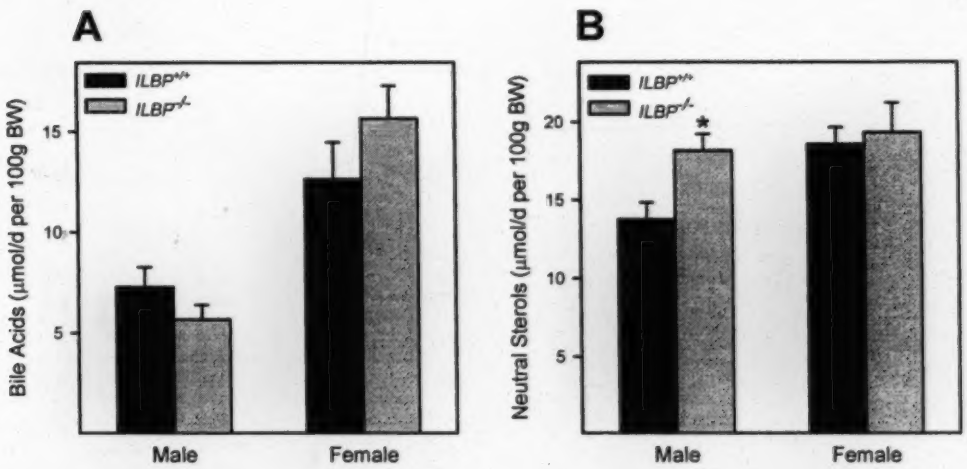

Figure 5-7. Bile acid and neutral sterol analysis in the fecal excretion of ILBP-deficient mice. Wild type and ILBP-deficient mice of both genders (n = 6 for each group) were kept in metabolic cages and fecal matter was collected every day for three days. Samples were desiccated, pulverized, extracted for lipids, and analyzed for bile acids (A) and neutral sterols (B) by colorimetric assay as described in section 5.2.4. Data shown are mean ± *SE*, expressed as µmol of bile acids or neutral sterols excreted per day normalizing body weight to 100g, and are a representative of 2 experiments. *, $P < 0.05$.

Absorption of lipid macronutrients in the gut is known to be regulated in response to both chronic exposures to higher intestinal lipid levels as well as each time a meal is ingested. Many lipid-regulated genes have been

identified and often each one has multiple strategies for gene regulation. The responses of these genes to lipid stimuli are complex and rely on gauging local lipid levels. If the lipid absorption in the intestine has been decreased in *ILBP* null mice as the data above suggest, ILBP may be important in acting as part of a bile acid sensor. In Figure 5-8, the amount of bile acids measured in whole small intestine extractions is higher in females compared to males, as expected due to known sex differences. However, this figure also shows that the *ILBP*$^{-/-}$ mice in both genders have lower levels of intestinal bile acids. The excretion findings in Figure 5-7 and the hepatic biliary output data in section 5.3.2 suggest that the amounts of bile acids entering the duodenum and leaving the animal are similar in *ILBP*$^{-/-}$ mice compared to their wild type counterparts. Therefore, it is likely that the purpose of ILBP may be to hold a certain level of bile acids within enterocytes, perhaps to aid in the gauging of lipids being absorbed and being passed to the blood stream. Mice lacking ILBP are able to conserve the enterohepatic cycling of bile acids but may have hampered bile acid or lipid perception, possibly affecting lipid dependent gene regulatory mechanisms. This may explain the putative decrease in lipid absorption and other phenotypes observed so far in *ILBP*$^{-/-}$ mice.

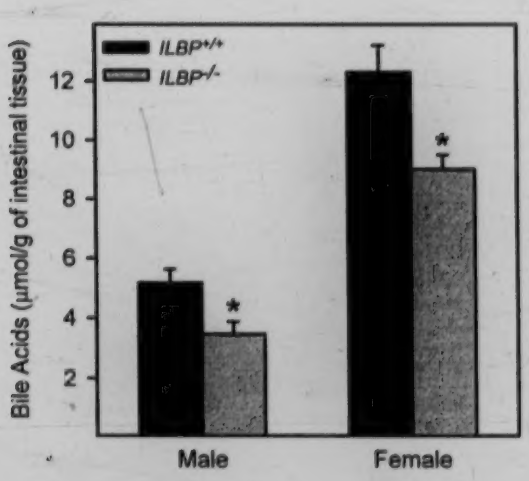

Figure 5-8. Determination of the bile acid content in small intestines from ILBP-deficient mice. Bile acids were isolated from pulverized small intestines after lipid extraction and analyzed using a diagnostic bile acid kit as described in section 5.2.4. Means (µmol bile acids/g wet weight of tissue) ± SE are shown for each group and gender (n = 5, each group) and are representative of 2 experiments.

5.3.4 Steady-state mRNA levels of genes involved in bile acid metabolism in ILBP-deficient mice

To identify possible compensatory mechanisms engaged in response to ILBP deficiency and a possible loss of dietary lipid absorption, mRNA levels for candidate genes involved in bile acid metabolism was assessed in the ileum and the liver. Hepatic and intestinal expression of specific genes were evaluated by comparative real time quantitative PCR in order to survey a larger set of genes. Conventional methods such as RNA hybridization blotting or RNase protection assays, though reliable, can be tedious if analyzing more than a handful of expressed genes. In order to test the methodology to be utilized in the *ILBP* null mice study, a gene with known

responses to specific stimuli was used to compare RNA hybridization blots to the real time PCR method to be used. Since *ILBP* is a well known FXR target whose mRNA expression responds dramatically to the presence of bile acids (Grober et al., 1999; Makishima et al., 1999), *ILBP* mRNA abundance was measured in mice challenged with bile acids. The Besnard group characterized the response of *ILBP* when challenged with bile acids both in tissue culture and in CD1 mice (Kanda et al., 1996; Kanda et al., 1998; Grober et al., 1999). In Figure 5-9A can be seen the typical response from CD1 mice as previously described and analyzed by RNA hybridization blotting: cholestyramine feeding reduces *ILBP* mRNA levels while TCA feeding increases *ILBP* mRNA abundance. Figure 5-9B illustrates results obtained using the same RNAs analyzed by real time PCR quantitative method and gave similar results. Note that the responses to the dietary challenges as determined by real time PCR are more pronounced. This is likely due to the underestimation of changes commonly observed with RNA hybridization blots because this method relies on autoradiogram densitometry to visualize and quantitate the results. In the last few years real time quantitative PCR has become routine and is known for its sensitivity and accuracy. Therefore, this methodology seemed satisfactory to survey the mRNA abundances of specific genes involved in bile acid metabolism from both the liver and the small intestine of ILBP-deficient mice.

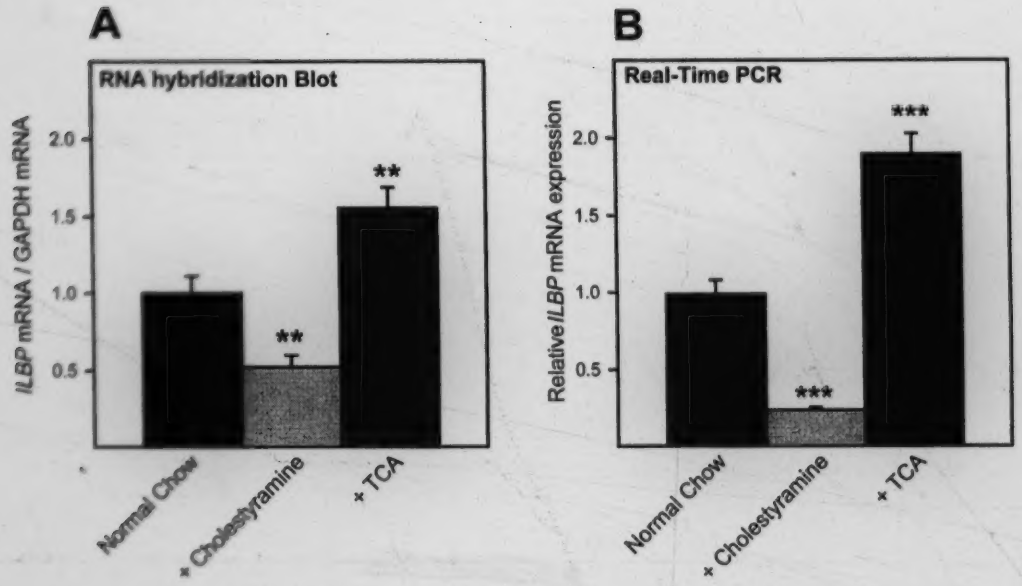

Figure 5-9. Quantitation of *ILBP* mRNA in CD1 mice. Three groups of female CD1 mice (n = 6 for each group) were fed normal chow or normal chow supplemented with either 2% cholestyramine or 0.5% TCA for 14 days. A, Levels of *ILBP* mRNA expression as quantitated by densitometry analysis of a RNA hybridization blot visualized by autoradiogram and normalized to *GAPDH*. B, Levels of ILBP mRNA expresion as quantitated by comparative real time quantitative PCR standardized to 18S rRNA. Data expressed are means ± SE are normalized to the normal chow fed group. **, $P < 0.01$; ***, $P < 0.001$.

The transcriptional regulation of genes involved in lipid metabolism is known to respond to dietary pressures. Examining the mRNA changes of specific genes in *ILBP*$^{-/-}$ mice may reveal changes in hepatic or intestinal lipid content based on known regulatory behaviours. In examining the expression patterns of specific genes from ileal tissue illustrated in Figure 5-10, it became apparent that the male model of ILBP deficiency differs markedly from the female version. As expected, the control glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) mRNA abundance was unchanged in

both genders and no *ILBP* mRNA was detected in *ILBP*$^{-/-}$ mice. However, gender disparity in ILBP-deficient mice was observed when comparing *L-FABP* mRNA levels in *ILBP*$^{-/-}$ mice and wild type mice. A small but significant decrease in male ileal *L-FABP* mRNA levels was observed. In contrast, female *ILBP*$^{-/-}$ mice displayed close to a 3-fold increase in *L-FABP* mRNA levels. Previous studies that have disrupted AP2 and L-FABP reported an increase in gene expression of intracellular lipid-binding proteins expressed in adipocytes and hepatocytes, respectively (Coe *et al.*, 1999; Martin *et al.*, 2003b). While the binding specificities of these other lipid-binding proteins can vary, it was thought that the compensatory upregulation of these proteins resulted in a phenotype-masking effect. The same may apply for the female *ILBP*$^{-/-}$ mice. Indeed, it is only the male *ILBP*$^{-/-}$ mice that exhibits cholesterol plasma decreases and increased neutral sterol excretion. L-FABP has a binding affinity for bile acids at least 1000-fold less compared to ILBP but still may play a direct or indirect role in cholesterol trafficking. Male *ILBP*$^{-/-}$ mice had 2-fold increases in *ABCa1* mRNA levels compared to their wild type counterparts (Figure 5-10A), while female mice exhibited only a modest increase (Figure 5-10B). It remains unclear whether ABCa1 is required to efflux cholesterol across the basolateral or apical membrane of the enterocyte (Mulligan *et al.*, 2003). However, there is some evidence that suggests that ABCa1 may be part of a group of ATP-binding cassette transporters that limit the intestinal absorption of sterols by excreting sterols back into the lumen on the apical side (Repa *et al.*, 2000b; Edwards *et al.*, 2002). The considerably higher levels of *ABCa1* mRNA in *ILBP*$^{-/-}$ male mice compared to female mice coincides with increased cholesterol excretion from males compared to no change in the female group (Figure 5-7). This may also explain the decrease in total plasma cholesterol in males while females remain unaffected (Figure 5-6). The expression of the other genes tested (*ASBT*, *tASBT*, *FXR*, and *LXR*) were unchanged.

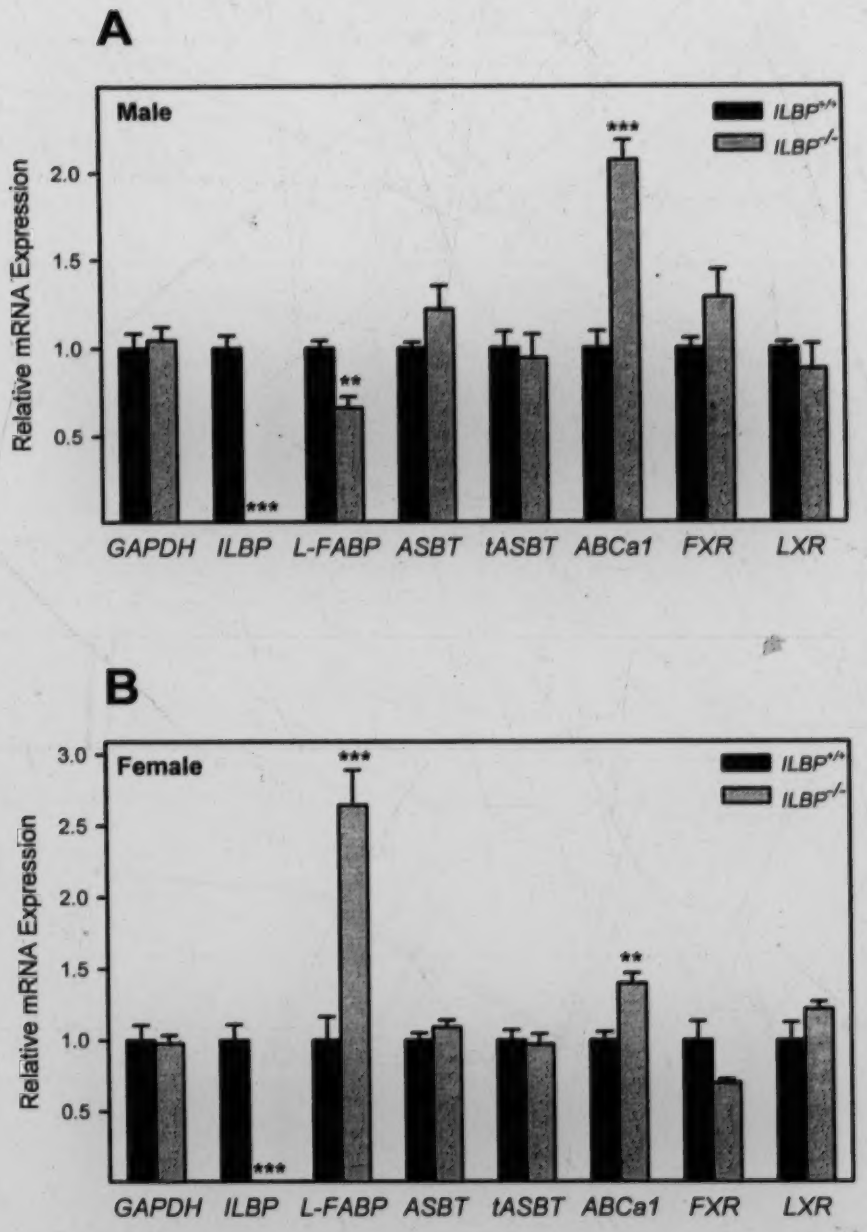

Figure 5-10. mRNA levels of specific genes involved with bile acid metabolism in ileums of ILBP-deficient mice. Total mRNA was isolated from the ileum section of the small intestine from *ILBP*$^{-/-}$ and wild type mice for both males (A) and females (B), transcribed into cDNA and subjected to real time PCR as described in Chapter 2. Data shown were determined using a comparative method standardized to 18S rRNA and shown as mean ± *SE*. Shown is an average of two experiments, $n = 6$ for all groups. **, $P < 0.01$; ***, $P < 0.001$.

To determine if liver lipids had been altered by ILBP deficiency, the mRNA expression of specific hepatic lipid-related genes were examined. Figure 5-11 shows a control *GAPDH* and four lipid transporters whose expression is known to be affected by bile acids. In both males (Figure 5-11A) and females (Figure 5-11B), the mRNA expression of *NTCP*, *BSEP*, and *ABCa1* are unaffected by ILBP deficiency. This further suggests that hepatic intracellular levels of bile acids are likely unchanged in *ILBP*$^{-/-}$ animals. However, hepatic *PLTP* expression appears to be decreased in *ILBP*$^{-/-}$ males while remaining unaffected in females. *PLTP* is a known LXR target (Laffitte *et al.*, 2003) and an overall decrease in body cholesterol suggested in section 5.3.3 could be responsible for a male specific decrease in *PLTP* mRNA expression. It should be noted that *ABCa1* expression is also induced by oxysterols through LXRα (Edwards *et al.*, 2002) yet paradoxically remains unaffected by a putative decrease in hepatic cholesterol. However, aside from the data in the previous section suggesting that hepatocyte intracellular bile acid concentration has decreased, the lipid levels in the livers of ILBP-deficient mice hve not been analyzed. Furthermore, while the effect of a singular stimulus on the expression of many genes involved in bile acid metabolism may have been relatively well characterized, the effect of simultaneous multiple stimuli on the promoters of these genes remains unknown. As such, it is difficult to predict which lipid changes could account for the observed mRNA abundance in ILBP-deficient mice.

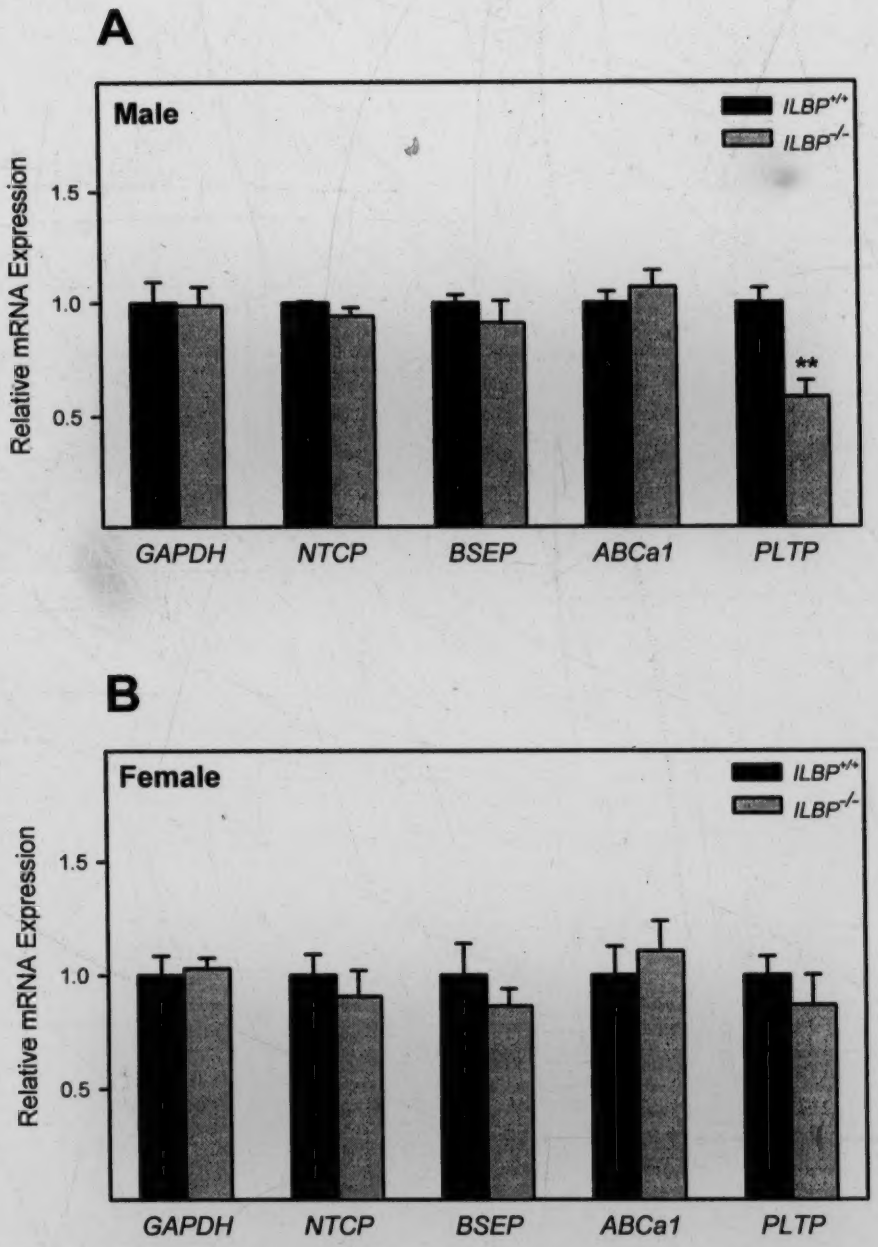

Figure 5-11. mRNA levels of specific lipid transporters in the livers of ILBP-deficient mice. Total mRNA was isolated from liver sections from *ILBP*$^{-/-}$ and wild type mice for both males (A) and females (B), transcribed into cDNA and subjected to real time PCR as described in Chapter 2. Data shown were determined using a comparative method standardized to 18S rRNA and shown as mean ± *SE*. Shown is an average of two experiments, $n = 6$ for all groups. **, $P < 0.01$.

Next, we examined mRNAs encoded by bile acid biosynthetic genes in the liver and our results were consistent with the data described earlier in this Chapter. For example, since ILBP deficiency has no effect on Cyp7a1 activity (Table 5-1 and Table 5-2), it is not surprising that *Cyp7a1*, *Cyp8b1*, and *Cyp27* mRNA expression is also unaffected in male (Figure 5-12A) and female (Figure 5-12B) *ILBP*$^{-/-}$ mice. An increased level of *HMGCR* was detected in male *ILBP*$^{-/-}$ mice but not in female *ILBP*$^{-/-}$ mice. This is consistent with the notion presented in section 5.3.3 that dietary cholesterol absorption in male mice may be less efficient while female cholesterol absorption is unaltered. These data are also consistent with the hepatic HMGCR activity in these animals (Table 5-1 and Table 5-2). An increased level of *HMGCR* mRNA and of HMGCR activity occurs in male ILBP-deficient mice, presumably to increase endogenous cholesterol synthesis and compensate for any putative loss of intestinal cholesterol absorption. While plasma cholesterol may be decreased in the male animals (Figure 5-6), these mice do not suffer from cholesterol depletion since biliary secretion of cholesterol is unhampered (Figure 5-3). *FXR* and *LXR* nuclear receptor mRNA expression remains unchanged in both *ILBP*$^{-/-}$ genders (Figure 5-12 A and B). However, *SHP* mRNA abundance has increased significantly in both male and female *ILBP*$^{-/-}$ mice. Since, as discussed in section 5.3.3 and as deduced from a lack of *Cyp7a1* transcriptional inhibition, hepatic bile acid levels remain unchanged in ILBP-deficient mice, the increase in *SHP* mRNA abundance cannot be due to bile acid induction. It is difficult to predict what stimulus or stimuli could have specifically increased *SHP* expression but these could potentially involve intracellular lipid levels besides bile acids. It is currently not known if other lipids affect hepatic *SHP* mRNA levels. The mechanism of increased *SHP* mRNA in both genders of *ILBP*$^{-/-}$ mice remains unclear.

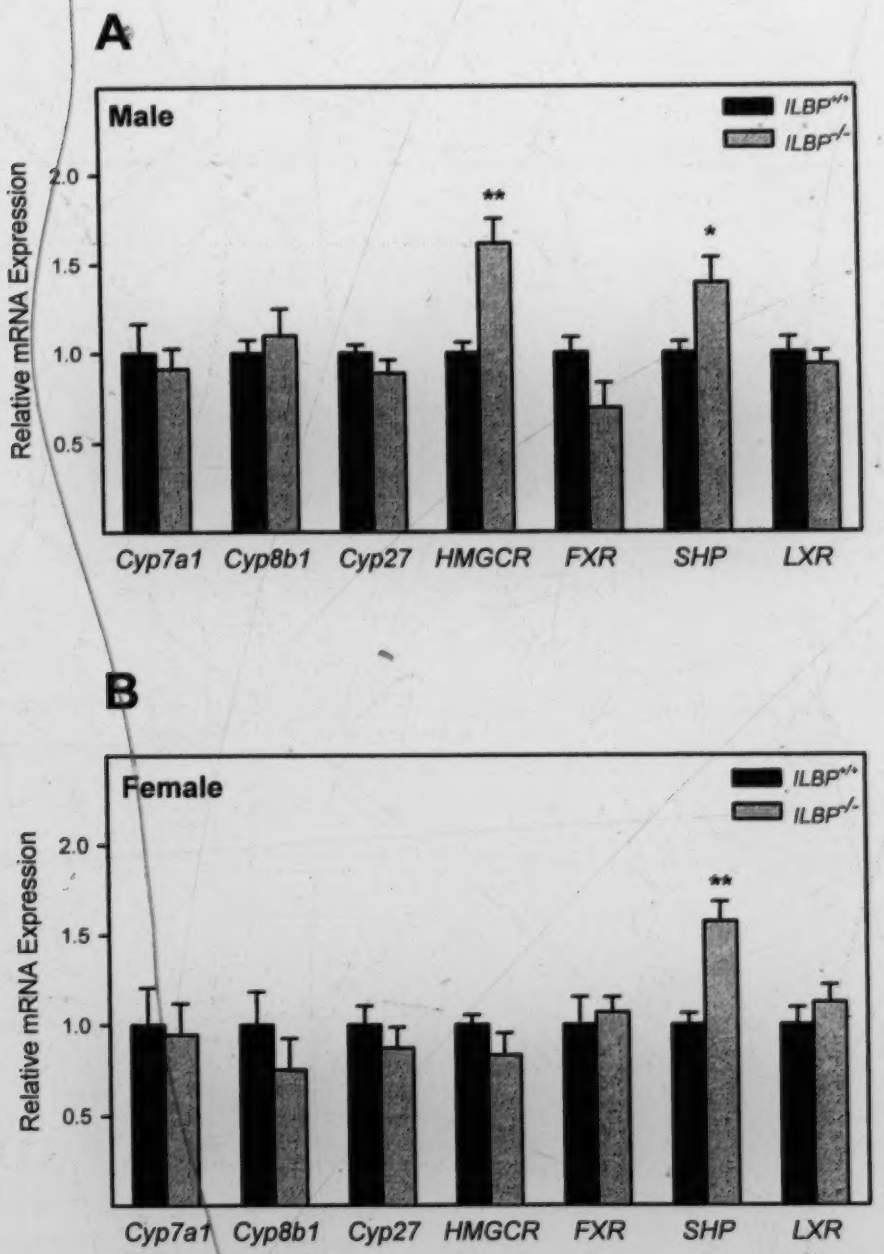

Figure 5-12. mRNA levels of specific genes involved with bile acid biosynthesis in livers of ILBP-deficient mice. Total mRNA was isolated from liver sections from *ILBP*$^{-/-}$ and wild type mice for both males (A) and females (B), transcribed into cDNA and subjected to real time PCR as described in Chapter 2. Data shown were determined using a comparative method standardized to 18S rRNA and shown as mean ± *SE*. Shown is an average of two experiments, $n = 6$ for all groups. *, $P < 0.05$; **, $P < 0.01$.

5.4 Discussion

To elucidate the function of ILBP, mice deficient in *ILBP* gene expression were studied. As discussed in Chapter 4, since ILBP binds bile acids preferentially and is the only known soluble protein expressed in the enterocyte to do so, the role of ILBP in the enterohepatic cycling of bile acids was specifically examined. Bile acids are required for the emulsification of dietary lipids and the inability to recycle or retain bile acids in a mouse should be readily apparent in simple lipid examinations. After years of breeding and studying the *ILBP*$^{-/-}$ mouse line, it is obvious that any phenotype involving a diminished capacity of bile acid retention will be subtle. The composition of bile, bile acid production, and the expression of bile acid biosynthetic enzymes from these animals revealed no change compared to wild type mice. These data strongly suggest that bile acids returning to the liver and, by extension, being absorbed from the ileum, were unaffected by ILBP deficiency. Therefore it does not appear that ILBP is necessary for the enterohepatic cycling of bile acids. However, inspection of other lipid characteristics in these animals did reveal that a shift in lipid metabolism had occurred. This led to the hypothesis that rather than being necessary for the intracellular transport of bile acids, ILBP serves as part of a bile acid sensor for compensation in lipid enzyme or transporter functions. This was first put forward when it was determined that ileal bile acid levels decreased in animals lacking *ILBP* (Figure 5-8). It has long been understood that while the liver may be the synthetic source of bile acids, the ileum also exerts enormous control over the enterohepatic circulation simply through the nature of bile acid reabsorption. Many organisms differ in enterohepatic cycling strategy merely by altering the level of importance of bile acid metabolic regulation in either the liver or the ileum (Shneider, 2001). ILBP

could play a role in gauging bile acids as they rapidly pass to the blood stream, by binding bile acids and allowing for their enterocytic perception.

Post-prandial intestinal bile acid concentrations are much higher than when the animal is in a starved state. In enterocytes, lipid absorption efficiency is adjusted depending on pancreatic hormonal regulation or on the contents of the intestinal lumen. This is accomplished through the upregulation of various transporters at varying levels of expression. The data presented in this Chapter suggest that *ILBP* null mice may have lower levels of cholesterol or triglycerides that are nevertheless not extreme enough to warrant noticeable body weight loss. Furthermore, a decrease in plasma cholesterol, higher levels of fecal neutral sterols, and a possible apical increase in the expression of *ABCa1* suggest that cholesterol absorption may not be as efficient in *ILBP* null mice as in wild type mice. One explanation may be that if ILBP-deficient mice are unable to monitor bile acid levels in the lumen as sensitively as normal mice, the enterocyte may sense only unfed levels of bile acids and may not induce maximal dietary lipid absorption. Therefore, since bile acids are constantly at unfed levels, enterocytes may respond by having only basal lipid absorption available. This basal level would be sufficient to absorb the necessary levels of dietary lipids but would never be at optimal efficiency. It is interesting to note that female ILBP$^{-/-}$ mice that express much higher levels of *L-FABP*, a weak bile acid binder, do not share the phenotype observed in males for cholesterol, who have a lower expression of *L-FABP* (Figure 5-10). This phenomenon of increased expression of other lipid binding proteins as compensation is also observed in mice deficient in AP2 (Coe *et al.*, 1999) and L-FABP (Newberry *et al.*, 2003). In order to confirm that there is a compensatory mechanism in place, it would be necessary to overwhelm this mechanism by feeding *ILBP*$^{-/-}$ mice bile acids or by crossing these mice with an L-FABP-deficient mouse line. The stress of additional bile acids may reduce phenotype masking and may reveal a protective function for ILBP. Another explanation is that without the

permissive binding capabilities of ILBP present, intestinal triglyceride and cholesterol transport may be reduced. However, this scenario is improbable since ILBP greatly prefers the binding of bile acids. Therefore, given the binding kinetics of ILBP and the lipid population in the ileum, most ILBP proteins would have its cavity filled with a bile acid molecule or molecules.

The characteristics of the liver, being the center of lipid homeostasis in the body, were also examined in ILBP-deficient mice. A mouse line deficient in the expression of a gene generates a model in which the importance of this gene can be assessed through phenotype inspection. In this case, given the propensity of ILBP to bind bile acids, the model to be studied was the enterohepatic circulation of bile acids. Since ILBP is not expressed in hepatocytes, examining the liver reveals what alteration the lack of ILBP in the ileum induces in the liver. If the return of bile acids to the liver via hepatic portal circulation was abrogated or diminished, bile acid secretion, bile formation, and bile acid biosynthesis would all be affected. Since all of these parameters in the *ILBP* null mouse were normal, it is more likely that the liver of these mice face a much different stress. Most notable is the increase in male hepatic HMGCR activity and mRNA in the male mice, while no change was apparent in the females. Considering this, as well as the decrease in HDL- and total plasma cholesterol and the increase in excreted neutral sterols, lower hepatic cholesterol levels are suspected due to poorer dietary cholesterol absorption. At first, the decrease in *PLTP* message in *ILBP*$^{-/-}$ males was thought to be due to lower levels of hepatic bile acids regulating *PLTP* through FXR (Urizar *et al.*, 2000). However, it is more likely that lower levels of cholesterol are acting on *PLTP* expression via LXR activation (Laffitte *et al.*, 2003). Hepatic triglycerides may also be lowered due to poorer lipid absorption in ILBP-deficient mice. The lower levels of plasma triglycerides, and presumably also decreased VLDL secretion, supports this possibility. For more proof, hepatic storage of triglycerides and fatty acid utilization would have to be examined in these animals. For example, lower

triglyceride storage in the liver could be indicated by a muted increase of fatty acid oxidation when *ILBP*$^{-/-}$ mice are fasted. Whether or not hepatic cholesterol or triglyceride levels have changed as a result of poorer lipid macronutrient absorption is unknown. However, the data in this Chapter suggest that bile acid levels are unaltered in ILBP-deficient mice.

The data in this Chapter support the idea that apical to basolateral enterocytic transport of bile acids is not dependent on ILBP. It is possible that these amphipathic molecules do not need a cytosolic transporter. An earlier study determined that the human bile acid-binding protein did not protect cultured cell lines from supraphysioligical levels of GCDCA and that cytotoxicity was induced under these circumstances (Torchia *et al.*, 2001). Therefore, the purpose of a bile acid binding protein may not be to serve as an intracellular transporter. Other mouse lines deficient in a lipid-binding protein exhibited more limited intracellular transport of bile acids but never to dangerous levels (Coe *et al.*, 1999; Newberry *et al.*, 2003). While increased expression of other lipid-binding proteins in the tissues of these mice may mask the extent of the phenotype, the fact remains that these proteins are not individually necessary. The function of these proteins is likely to improve the interaction of relatively hydrophobic molecules in a hydrophilic world. Certainly L-FABP has been shown in hepatocytes from *L-FABP* null mice to aid in the delivery of fatty acids to peroxisomes for branching (Atshaves *et al.*, 2004), storage and VLDL secretion (Newberry *et al.*, 2003), and overall cellular distribution (Martin *et al.*, 2003a, b). Another function for lipid-binding proteins may be to deliver lipids to the nucleus for nuclear receptor perception. This was first proposed by the Schroeder group for L-FABP. L-FABP was found to target fluorescent fatty acids to the nucleus of the hepatocyte and to nuclear receptors (Huang *et al.*, 2002; Huang *et al.*, 2004). It is possible that ILBP could retain bile acids in the cytosol while not slowing overall bile acid absorption so that intracellular levels could be maintained at a higher level and allow for a more sensitive reaction of compensation

mechanisms. Another explanation could be, as Schroeder's group implies for L-FABP, that ILBP could be delivering bile acids to the nucleus for FXR loading. Therefore, the absence of ILBP would delay FXR activation, thus affecting transcriptional regulation of genes involved in bile acid metabolism. In this regard it is noteworthy that *ASBT* expression is indirectly regulated by FXR activation (Chen *et al.*, 2003) and *LXR* expression is directly activated by FXR (Fitzgerald *et al.*, 2002). Since both *ASBT* and *LXR* mRNA levels are unchanged in *ILBP* null mice despite the detection of lower bile acids in the small intestine, FXR may require the presence of ILBP in the enterocyte to function normally. However, other lipid transcriptional pressures are likely in effect in the enterocytes of these animals and it is impossible to predict from this study what those may be.

In conclusion, characterization of the *ILBP* null mouse provides evidence that suggests a role of ILBP in intestinal lipid absorption and, by extension, lipid homeostasis. The evidence in this study reveals that the volume of bile acids being delivered to the liver during their enterohepatic cycling appears to be largely unaffected by the absence ILBP. Rather, ILBP appears to function in a role of regulating the absorption and transporters of other lipids, probably in concert with L-FABP and possibly other proteins. This is likely accomplished through the retention of bile acids in the enterocyte, against the fast current of bile acid delivery to the blood stream, in order to allow for the gauging of intracellular bile acids to enable appropriate compensatory mechanisms. Furthermore, this study illustrates the interconnectivity of many classes of lipids and suggests that their metabolism, in this case as related to intestinal absorption.

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Chapter 6

Summary and Conclusions

The liver plays a key role in regulating cholesterol homeostasis and is the major organ involved in cholesterol elimination from the body. This is accomplished by secreting cholesterol into bile either in the form of free cholesterol or as bile acids. Highly insoluble molecules of cholesterol are converted to bile acids, a relatively more soluble and amphipathic class of lipids, in the liver through a series of chemical modifications. Bile acids are natural detergents that are an indispensable component of the digestive system. Their levels are highly conserved in the body in an effort to ensure that an optimal concentration is present in the gut for the emulsification and absorption of lipophilic nutrients. As part of bile, bile acids are released into the intestine and, after aiding in the solubilization and absorption of dietary lipids largely in the jejunum, they are themselves absorbed at the level of the ileum. Although unconjugated bile acids are passively reabsorbed along the whole of the small intestine, the majority of bile acids are reclaimed actively in the ileum where the Na^+/bile acid cotransporter ASBT has been identified and characterized (Wong *et al.*, 1994; Shneider *et al.*, 1995). Once these bile acids are absorbed, they are returned to the liver for reuse. This conservation of bile acids is known as the enterohepatic circulation of bile acids. Bile acids represent the major method of cholesterol elimination from the body and the absorption of these natural detergents is tightly regulated. Therefore, understanding the ileal bile acid metabolism can provide important insights in to how sterols and their derivatives affect whole body physiology.

The cloning and characterization of genes of putative bile acid binding and transporting proteins ten years ago started a new period in the study of molecular mechanisms within the enterohepatic circulation. As a result of these studies, much is known about the transporters that take up or secrete bile acids in various tissues. However, lipid-binding proteins such as ILBP, though long thought to be implicated in intracellular trafficking, are not as well understood. Only recent studies from *L-FABP* null mice or hepatocytes derived from these animals have started to unveil what functions lipid-binding

proteins may contribute to lipid metabolism (Wong *et al.*, 1994; Shneider *et al.*, 1995; Martin *et al.*, 2003a, b; Newberry *et al.*, 2003; Atshaves *et al.*, 2004). Using these studies on the promiscuous binding L-FABP to extrapolate and shed light on the function of ILBP in the enterohepatic circulation of bile acids is difficult at best. As such, the molecular events that govern the cellular movements and interactions of bile acids once they are taken up by enterocytes of the small intestine remain a virtual black box. The deficiency of models elucidating the role ILBP may play in the translocation of bile acids from the cellular sites of uptake to the sites of efflux in ileocytes has hindered progress in this area.

The first objective of this thesis was to establish a reliable, expeditious, and provident method of multiple bile acid analyses. An initial assessment of available chromatographic methods for bile acid analysis revealed that the very nature of bile acids posed a problem in achieving this goal. Bile acids are an amphipathic class of molecules that can greatly differ in hydrophobicity and other physicochemical properties compared to other varieties of lipids. As such, available methods for bile acid analysis were either complex and involved multiple procedures or facile but insensitive. Therefore, the approach taken was to take a simple method and improve sensitivity, analysis time, and reliability. HPLC methods employing an evaporative light scattering detector (ELSD) were by far the most sensitive and cost-effective (Novakovic *et al.*, 1998; Roda *et al.*, 1998; Criado *et al.*, 2003). Generating an isocratic solvent system to resolve all varieties of bile acids employing an HPLC coupled to an ELSD satisfied the criteria of improvements listed above. Generating this methodology of bile acid analysis provided a necessary instrument for the description of ILBP bile acid-binding capabilities and for a characterization of the potential functions of this protein. This HPLC method has already proven useful in the characterization of bile in the human transgenic Cyp7a1 mouse (Drover and Agellon, 2004).

Another objective of this thesis was to determine if murine ILBP has a preference for specific species of bile acids or fatty acids. The approach utilized was a competition binding assay where equal amounts of lipids were incubated with recombinant murine ILBP. Protein and free lipids were separated by gel filtration and lipids associated with the protein were analyzed. Among fatty acids, murine ILBP preferred species that had longer chain length and increased saturation, similar to other members of the intracellular lipid-binding protein family. Among the bile acids, murine ILBP showed the greatest preference for conjugated species that contained a doubly hydroxylated steroid moiety. Since the speciation of bile acids can vary between organisms, it is not surprising that the binding preferences for ILBP expressed in mice are different from those expressed in humans or rats (Zimmerman *et al.*, 2001; Tochtrop *et al.*, 2003). The goal of the ILBP binding study described in this thesis was to determine, in simple terms, a binding preference ILBP may have for two classes of lipids. However, given that ILBP has two cooperative binding sites for bile acids as determined by recent NMR and kinetic studies (Tochtrop *et al.*, 2003), the competitive binding assay may be an oversimplification of physiological events. Indeed, the ileocyte may have a large range of bile acid intracellular levels depending on the fed state of the animal. Thus, the binding preference of ILBP for various bile acid species may change for each binding pocket as the bile acid levels do within the cell. Furthermore, usually in a given bile acid pool of any animal, there will be only 2 to 3 prevalent bile acid species. Thus, it may only matter how ILBP deals with these more common bile acids. Therefore, depending on which bile acid species can potentiate FXR activation more effectively, the binding preference of ILBP may play a role in FXR-mediated gene regulation. This proposed mechanism may allow for the "fine tuning" of the bile acid pool by allowing for the regulated intestinal absorption of specific bile acid species.

The last goal of this thesis was to determine if expression of ILBP was essential for the conservation of bile acids and what role it may play in the enterohepatic circulation. For over two years mice lacking ILBP were bred for experimental analysis. The *ILBP*$^{-/-}$ null mice were viable and indistinguishable from their wild type counterparts. Plasma lipids in these animals had been altered but not in a manner consistent with bile acid depletion. Furthermore, examination of liver functions, including bile formation and Cyp7a1 activity, were completely unaffected by a lack of ILBP. From these data, it was easy to surmise that the bile acid pool in the *ILBP*$^{-/-}$ mice had not been diminished. However, hepatic HMGCR activity was significantly increased in males concomitant with an increase in fecal neutral sterols. Coupled with the male-specific decrease in total plasma cholesterol, the notion of decreased intestinal cholesterol absorption was put forth. An increased *ABCa1* mRNA abundance detected only in males is consistent with the suggestion of a previous study, that apical ABCa1 is involved in a futile secretion of cholesterol in enterocytes and a reduction of cholesterol absorption (Repa *et al.*, 2000). The nearly 3-fold increase in *L-FABP* mRNA was also suggested to protect the female *ILBP*$^{-/-}$ mice from similar cholesterol absorption problems. Similar to other studies involving the ablation of genes encoding for lipid-binding proteins, a compensatory increase in expression of other intracellular lipid-binding proteins may produce a masking of phenotype. Since both *ILBP*$^{-/-}$ genders also have decreased plasma triglyceride levels, the suggestion was made that fatty acid uptake could also be impaired in these animals. However, neither gender experienced any body weight decreases associated with loss of dietary lipid absorption. Thus, the putative reduced intestinal lipid absorption would be considered subtle. Since intestinal bile acids had decreased in both genders, it was presumed that lower intracellular bile acid levels triggered the problematic intestinal lipid absorption. The data from this model, therefore, suggest that a possible function of ILBP is to preserve increased bile acid levels in the ileum to aid in

bile acid perception and consequently the regulation of lipid metabolism. A possible mechanism for this hypothesis would be an interaction of ILBP with FXR in a ligand loading scenario. It is noteworthy that a phenotype in the *ILBP* null mouse is apparent without stressing the animal (i.e. through bile acid feeding), unlike most other intracellular lipid-binding protein deficient mice (Haunerland and Spener, 2004). Though the phenotype may not have the expected effect on bile acid metabolism, the interconnectivity of lipid metabolism in the enterocyte may explain the observed change in cholesterol and triglyceride metabolism. Furthermore, the gender disparity that exists in the *ILBP* null mice allows for two separate models of study for ILBP functionality and underlines a difference in lipid metabolic strategy in the enterocyte and perhaps the liver inherent in the sexes.

The role of ILBP in ileal recovery of bile acids may be subtle. The data in this thesis show that ILBP is not necessary for the conservation of bile acids in the enterohepatic circulation, nor is there any evidence of a diminished capacity to recover bile acids. This differs from studies of mice lacking other intracellular lipid-binding proteins, where a reduced capacity of intracellular trafficking of lipids is accompanied by a lowered ability for lipid utilization. While the data in this thesis do not demonstrate any loss of intracellular transit of bile acids, intestinal bile acid levels appear to be decreased. This coupled with the apparent modification in lipid metabolism in these animals, implies a role for ILBP as part of a bile acid barometer in the ileocyte. It is possible that without ILBP, *ILBP*$^{-/-}$ mice may have impaired acuity of bile acids and are never able to detect fed levels of bile acids in the intestine. This may, in turn, not allow for the potentiation of intestinal lipid absorption associated with an animal in a fed state and maximal dietary lipid absorption is never achieved.

Other questions regarding ILBP function and *ILBP* null mice will be addressed role in future studies. As discussed in Chapter 1, ASBT has been reported to form large complexes with ILBP (Kramer *et al.*, 1993; Kramer *et*

al., 1995; Kramer *et al.*, 1998). Given that ASBT can still function despite the lack of ILBP, what function do these complexes serve? Intestinal glucose transporters (Kelada *et al.*, 1992; Stephens and Pilch, 1995; Kellett, 2001) and NTCP (Mukhopadhayay *et al.*, 1997) have been shown to translocate from internal cellular vesicles to the plasma membrane in response to increased intracellular concentrations of their corresponding substrate via cell signaling pathways. Similar to these transporters, is there translocation of ASBT in a mechanism in which bile acid uptake can be feedback regulated and is it dependent on complexing with ILBP? What are the major bile acid-binding proteins in *ILBP*$^{-/-}$ mice? Can increased expression of L-FABP mask the phenotype in *ILBP* null mice? What is the role of ILBP in FXR-mediated gene regulation? By what mechanism can bile acid levels in the intestine affect dietary lipid absorption? By what method do bile acids translocate in ileocytes? Is a chaperone necessary? What is the rate limiting step in the molecular events surrounding bile acid absorption in the ileum? Future studies should address these issues.

In conclusion, the roles of cytosolic ILBP with respect to intracellular bile acid trafficking and in the enterohepatic circulation appear to be subtle. The challenge of future studies will be to determine the mechanism of bile acid vectorial transport across the ileocyte during intestinal absorption. Given the great variation of bile acid species, ILBP expressed in different organisms is likely tailored to the bile acid pool that the animal possesses. In this manner, the biological activities of certain varieties of these molecules could be elaborated by the binding affinity ILBP has for bile acids. Figure 6-1 summarizes the molecular events following bile acid uptake by an enterocyte in the ileum. Bile acids in the lumen of the small intestine undergo active transport via membrane-bound ASBT. Internalized bile acids likely interact with ILBP but directional trafficking of bile acids from the apical to basolateral side does not require ILBP. The ultimate fate of the bile acids absorbed from the lumen is to be secreted into circulation by a membrane-bound protein,

possibly tASBT, to eventually return to the liver via the hepatic portal circulation. Meanwhile, ILBP expressed in the ileocyte likely retains a basal population of intracellular bile acids. The presence of ILBP may allow for sensitive regulation of bile acid-dependent mechanisms within the ileocyte to maintain lipid homeostasis. Therefore, through ILBP, bile acids may regulate cellular functions such as the expression of FXR-dependent genes. Whether ILBP enhances FXR activity is yet to be determined. However, ILBP could accomplish this indirectly by merely maintaining perceptible intracellular bile acid levels or directly through interaction with FXR and ligand loading. While ILBP may not be necessary for intestinal absorption of ILBP, it may be part of a modulating mechanism that affects whole body lipid homeostasis.

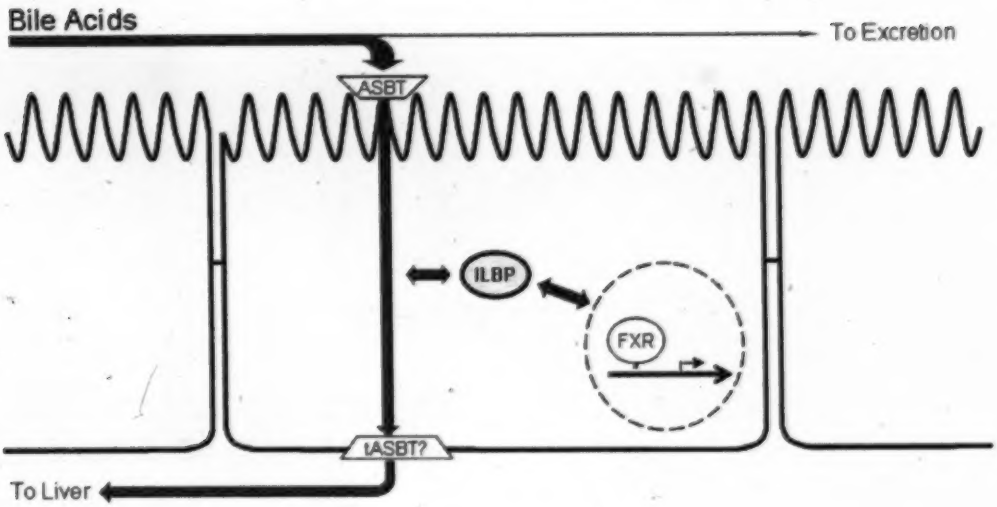

Figure 6-1. Schema depicting the molecular events that occur once bile acids are internalized by ileocytes. Bile acids in the lumen of the ileum, which have been secreted as part of bile to emulsify dietary lipids, undergo active transport via ASBT on the apical side. Bile acids are transported from the site of uptake and are secreted basolaterally through an unknown transporter, tASBT being a potential candidate. This vectorial transit across the ileocyte to the sites of efflux is independent of ILBP. Bile acids are in equilibrium with ILBP retaining a population of these molecules in the ileocytes. ILBP may play a subtle role in intracellular trafficking of bile acids leading to interaction with other cellular proteins such as FXR to modulate FXR-mediated gene regulation and cellular function.

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Appendix A

Expression of Cholesterol 7α-Hydroxylase Restores Bile Acid Synthesis in McArdle RH7777 Cells

A version of this chapter has been published: Labonté, E.D., Li, Q., Agellon, L.B. (2000) *Arch. Biochem. Biophys.* **381**, 273-277.

Introduction

The bile acid biosynthesis is the major catabolic route for cholesterol. The classic or neutral bile acid synthetic pathway is initiated and completed in the liver and uses cholesterol as substrate. The enzyme cholesterol 7α-hydroxylase (Cyp7a1) catalyzes the first and rate-limiting step in this pathway [reviewed in (Russell and Setchell, 1992; Vlahcevic *et al.*, 1999)]. The alternate or acidic bile acid biosynthetic pathway accepts cholesterol with a hydroxylated side chain as substrate (Wachtel *et al.*, 1968; Anderson *et al.*, 1972; Axelson and Sjovall, 1990; Schwarz *et al.*, 1996). The contribution of the alternate pathway can range from ~3 to ~50% of the total bile acids synthesized by the liver, depending on the species (Lund *et al.*, 1996; Vlahcevic *et al.*, 1997; Duane and Javitt, 1999). In total, at least 16 enzymes are involved in the conversion of cholesterol to primary bile acids.

Cyp7a1 gene expression declines rapidly in cultured hepatocytes (Hylemon *et al.*, 1985; Princen *et al.*, 1989; Hylemon *et al.*, 1992; Ellis *et al.*, 1998). In many liver-derived cell lines, cytochrome P450 expression is difficult to maintain. As a result, Cyp7a1 expression is either very low or absent. Correspondingly, the production of bile acids is decreased or undetectable in these cells. Studies of Cyp7a1 and bile acid formation have thus far mostly been limited to human hepatoma HepG2 cells. This tumor cell line, however, is far from ideal because it produces C_{27} sterol bile acids due to impaired oxidation and side chain cleavage (Everson and Polokoff, 1986; Axelson *et al.*, 1991; Pandak *et al.*, 1996). Furthermore, HepG2 cells differ from normal human hepatocytes with regard to their conjugation of bile acids (Cooper *et al.*, 1994) and the efficiency of bile acid production is much lower than that of normal hepatocytes (Javitt *et al.*, 1989). The synthesis of bile acids involves a variety of enzymes in addition to Cyp7a1 (Russell and Setchell, 1992; Vlahcevic *et al.*, 1999) and it is not known if the expression of

genes encoding these enzymes also undergoes extinction. Previous studies have shown that McArdle RH7777 cells do not express Cyp7a1 (Dueland *et al.*, 1997) but may retain much of the bile acid biosynthetic pathway due to the origin of the cell line. A McArdle RH7777 cell line expressing Cyp7a1 may serve as a useful model for the study of bile acid formation. Therefore, in this study, I determined whether simple restoration of Cyp7a1 activity would be sufficient to allow for the complete synthesis of bile acids in McArdle RH7777 rat hepatoma cells.

Methods

Cell culture and construction of the Cyp7a1 expression plasmid

The McArdle RH7777 cells were maintained using standard tissue culture techniques (Freshney, 1994). All cell lines in this study were adherent and were cultured in dishes of different sizes (35 to 100 mm). Cells were maintained at 37°C and 5% CO_2 and routinely passaged every second day by washing with PBS, treating briefly with 0.25% trypsin solution (Life Technologies, Burlington, ON) and pipeting several times in culture medium containing serum. Cell number was determined by use of a hemocytometer and approximately 5 x 10^5 cells were plated in 100 mm dishes. McArdle RH7777 cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 4500 mg/L glucose, 10% fetal bovine serum (FBS), and 10% calf serum (CS), without sodium pyruvate. For frozen storage, cells were collected and resuspended in DMEM containing 10% FBS and 10% dimethyl sulfoxide. Cells were aliquoted in to 1.5 ml cryovials and placed at -70°C in an isopropanol cooler (Stratagene, La Jolla, CA). After 16 h, vials were transferred to liquid nitrogen for long term storage.

The Cyp7a1 expression vector was constructed by Q. Li. A derivative of the pCEP4 expression vector (Invitrogen Corp., Carlsbad, CA) was made by removing the *Cla*I/*Eco*RV restriction fragment. This modification eliminated the ability of the plasmid to replicate autonomously and therefore allowed the selection of stable integrants by screening for hygromycin B resistance that is encoded by the plasmid. A cDNA fragment corresponding to the entire translated region plus portions of 5'- and 3'-untranslated regions of the rat *Cyp7a* mRNA (Jelinek *et al.*, 1990) was cloned downstream of the CMV promoter of the modified pCEP4 expression vector and transfected into McArdle RH7777 cells using Clonfectin (Clontech Laboratories Inc., Palo Alto, CA).

Analysis of clones transfected with the Cyp7a1 expression plasmid

Clones surviving the hygromycin B (Sigma–Aldrich Canada Ltd., Oakville, ON) selection were picked and screened. The genomic DNA was analyzed for the presence of the expression vector by *in vitro* DNA amplification using a primer pair that specifically amplifies the recombinant *Cyp7a1* gene. Total RNA from both parental and recombinant cells was purified according to a standard procedure (Chomczynski and Sacchi, 1987) which is described in Chapter 2. Detection of *Cyp7a1* mRNA was tested by RNA hybridization blot, also described in Chapter 2. Briefly, the extracted total RNA (30 µg) was separated by agarose gel electrophoresis, transferred to Hybond-N membrane (Amersham Pharmacia Biotech, Baie d'Urfé, QC), and hybridized with ^{32}P-labeled rat Cyp7a1 cDNA. The membrane was autoradiographed after washing under stringent conditions. Clones showing the presence of the recombinant *Cyp7a1* mRNA were expanded. The sterol 27-hydroxylase (Cyp27) mRNA was detected by *in vitro* DNA amplification.

Total RNA isolated from rat liver and McArdle RH7777 cells was reverse transcribed to cDNA using random primers. Two sets of primer pairs were used to amplify the 5' and 3' regions (nucleotides 430–1210 and 1091–2065, respectively; GenBank Accession No. M73231) (Shayiq and Avadhani, 1992) of the rat *Cyp27* mRNA. A portion of the glyceraldehyde-3-phosphate dehydrogenase (G3PDH) mRNA was also amplified as a control.

Cyp7a1 microsomal protein levels and activities in cultured cell lines were measured by Q. Li. Briefly, microsomes from cells were prepared in buffer containing protease inhibitors (aprotinin, 2 mg/ml; EDTA, 1 mM; phenylmethylsulfonyl fluoride, 100 mg/ml; leupeptin, 1 mg/ml). The protein concentration of microsomal preparations was determined by the Lowry method (Lowry *et al.*, 1951), using bovine serum albumin as the standard. The recombinant Cyp7a1 protein was detected using an immunoblot procedure similar to that described in Chapter 2. Briefly, a chicken antibody was raised against a peptide corresponding to the carboxylterminal amino acid sequence of mouse Cyp7a1 (Agellon and Cheema, 1997). The antibody used in immunoblots was preadsorbed to McArdle RH7777 total cell lysates. Microsomal preparations (100 µg total protein/assay) were measured for Cyp7a1 activity (Martin *et al.*, 1993; Agellon, 1997). For substrate, [^{14}C]cholesterol (10 mCi/mmol; 13 nmol per assay; Amersham Pharmacia Bbiotech) encapsulated by β-cyclodextrin (Sigma-Aldrich Canada Ltd., Oakville, ON) was used in these assays. Reaction products were separated by TLC using an ethyl acetate-toluene (3:2 v/v) solvent system. The amount of radioactivity in the spots corresponding to 7-hydroxycholesterol was quantitated using a Fuji BAS1000 plate imager. Calculation of enzyme activity took into account the dilution of the exogenous labeled cholesterol by the endogenous cholesterol in the microsomes.

Detection of bile acids in the cell culture medium

Radiolabeled cholesterol substrate was prepared by encapsulating [^{14}C]cholesterol and unlabeled cholesterol in β-cyclodextrin. Confluent cultures (100-mm dishes) of parental and recombinant cells were incubated in medium containing the radiolabeled cholesterol substrate for 16 h. After incubation, the medium was removed and the cells were washed with PBS. The cells were overlaid with fresh culture medium (10 ml per dish) without the radiolabeled substrate and then returned to the incubator. After 16 h, the culture medium was collected, loaded onto a Speed C18/14 cartridge (Applied Separations, Allentown, PA) for lipid extraction (Setchell and Worthington, 1982). The bound material was eluted from the column and then analyzed by TLC along with pure bile acid standards described in chapter 2. Pure 7α-hydroxycholesterol was purchased from Steraloids (Newport, RI). The TLC plate was developed in an isoamyl acetate:propionic acid:1-propanol:water (4:3:2:1) solvent system (Van den Ende *et al.*, 1983). The radioactive materials on the TLC plate were visualized by autoradiography and their mobilities were compared with those of the bile acid standards visualized by iodine staining. In some experiments, the material in selected bands was recovered from the TLC plate for further analysis. Determination of molecular mass by mass spectrometry was done at the University of Alberta Mass Spectroscopy Laboratory.

Results

Expression of Cyp7a1 in McArdle RH7777 cells

Cyp7a1 catalyzes the initial step in the conversion of cholesterol into bile acids in liver cells. McArdle RH7777 is a hepatoma cell line that was known to not produce bile acids or express Cyp7a1 (Dueland *et al.*, 1997). Initial investigations confirmed that Cyp7a1 mRNA is not detectable in total RNA extracted from McArdle RH7777 cells analyzed by ribonuclease protection assay or *in vitro* amplification of Cyp7a1 cDNA. Accordingly, microsomes purified from McArdle RH7777 cells do not contain cholesterol 7α-hydroxylation activity that is significantly higher than that in microsomes of cells that are known to be devoid of Cyp7a1 activity, such as cells of nonhepatic origin.

Bile acid synthesis is complex and involves a variety of enzymes in liver (Russell and Setchell, 1992; Vlahcevic *et al.*, 1999). While McArdle RH7777 cells do not express Cyp7a1, the remainder of the bile acid biosynthetic pathway may be expressed in McArdle RH7777. To determine if restoration of Cyp7a1 activity is sufficient to reestablish bile acid synthesis in McArdle RH7777 cells, an expression vector featuring the entire sequence encoding the rat Cyp7a1 under the control of the constitutive CMV promoter was constructed. The McArdle RH7777 cells were transfected with the expression vector and colonies that stably integrated the exogenous DNA were isolated. Analysis of total RNA and microsomal proteins indicated that the recombinant Cyp7a1 mRNA (Figure A-1A) and protein (Figure A-1B) were detectable only in cells transfected with the Cyp7a1 expression plasmid. The 7α-hydroxylation activity in purified microsomes was comparable to that in microsomes isolated from the livers of C57BL/6J mice fed a cholestyramine-containing diet (Figure A-2). Moreover, the amount of

Cyp7a1 activity in the assay was dependent on the amount of recombinant microsomes used. No significant activity was detected in the microsomes of the parental McArdle RH7777 cells. The cholesterol 7α-hydroxylation activities of microsomes isolated from various Cyp7a1-expressing clones ranged from ~0.5- to ~6-fold (8–120 pmol/min/mg of protein) compared to that in microsomes isolated from livers of chow-fed C57BL/6 mice. A McArdle RH7777 cell line that stably expressed Cyp7a1 was therefore established.

Figure A-1. Analysis of recombinant Cyp7a1 gene expression in rat hepatoma cells. (A) Detection of the recombinant Cyp7a1 mRNA by RNA hybridization blot. (B) Immunoblot detection of the recombinant Cyp7a1 in purified microsomes. Lanes 1 and 2, RNA (A) or protein (B) from parental McArdle RH7777 cells; lane 3 and 4, RNA (A) or protein (B) from vector transfected McArdle RH7777 cells.

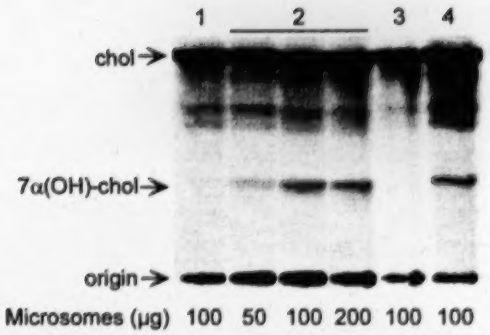

Figure A-2. Analysis of Cyp7a1 activity in rat hepatoma cells. The amount of microsomes (μg of total microsomal proteins) used in Cyp7a1 activity assays is indicated below each lane. Lane 1, parental McArdle RH7777 microsomes; lane 2, various amounts of microsomes from recombinant cells; lane 3, boiled microsomes from recombinant cells; lane 4, microsomes purified from livers of mice fed with 2% cholestyramine chow. 7α-hydroxycholesterol (7α(OH)-chol) and cholesterol (chol) are indicated on the left.

Characterization of McArdle RH7777 cells expressing Cyp7a1

To determine if the recombinant McArdle RH7777 cells were capable of completing bile acid synthesis from cholesterol, cultures of both parental and recombinant cells were incubated with [^{14}C]cholesterol, washed, and then reincubated in fresh culture medium. It was previously demonstrated that bile acid treated McNtcp cells, which are McArdle RH7777 cells expressing the sodium/taurocholate co-transporting polypeptide (NTCP), were capable of secreting bile acids into the culture medium (Torchia *et al.*, 1996). Therefore, the conditioned media of both parental and recombinant cells were analyzed for the presence of bile acids. The conditioned medium of the Cyp7a1-expressing cells (Figure A-3, right lane), but not that of parental cells (Figure A-3, left lane), contained radiolabeled compounds whose mobilities

on TLC plates corresponded to those of pure taurine-conjugated and free forms of dihydroxy and trihydroxy bile acids. Thus, expression of Cyp7a1 is by itself sufficient to restore the bile acid biosynthetic pathway in McArdle RH7777 cells.

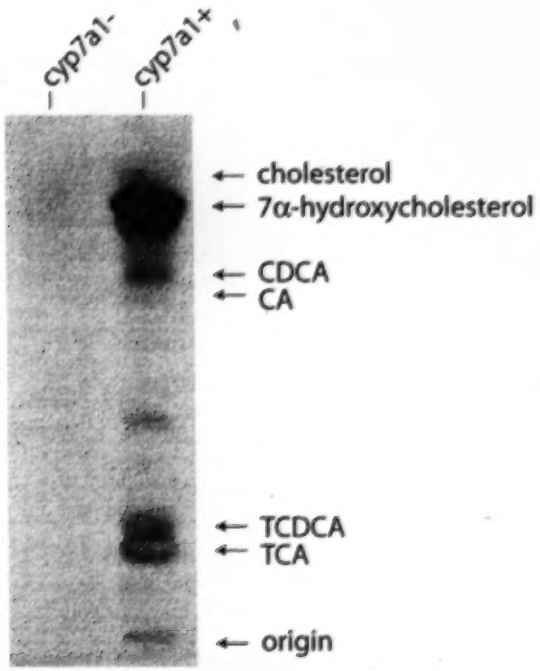

Figure A-3. Detection of bile acids in the culture medium of Cyp7a1-expressing hepatoma cells. The synthesis of bile acids from cholesterol was assessed by the following the fate of [^{14}C]cholesterol. The conditioned medium of cholesterol-loaded cells was lipid extracted, developed by TLC, and visualized by autoradiography. Cyp7a1-, sample extracted from parental cells; Cyp7a1+, sample extracted from Cyp7a1-expressing McArdle RH7777 cells. The mobilities of bile acids (CA, cholic acid; CDCA, chenodeoxycholic acid; TCA, taurocholic acid; TCDCA, taurochenodeoxycholic acid), 7α-hydroxycholesterol, and cholesterol are indicated on the right.

Interestingly, the most abundant radiolabeled species in the conditioned medium of Cyp7a1-expressing cells that was revealed by TLC did not

correspond to the common bile acid species. The unknown compound was therefore recovered from the TLC plate and further analyzed. The compound comigrated with authentic 7α-hydroxycholesterol (R_f = 0.824 ± 0.002; n = 6 determinations) by TLC. Mass spectrometric analysis revealed a molecular mass of 402.6, corresponding to hydroxylated cholesterol. Further, the mass spectrometric analysis also revealed that the compound recovered and authentic 7α-hydroxycholesterol has identical disconnection patterns. Therefore, while bile acids are produced by the recombinant cell line, the most abundant sterol product secreted after [^{14}C]cholesterol treatment was identified as the immediate product of Cyp7a1 catalysis, 7α-hydroxycholesterol.

The observation that McArdle RH7777 cells are unable to convert cholesterol into bile acids via the alternate bile acid biosynthetic pathway would suggest that these cells are either impaired or deficient in Cyp27 activity. To determine if the *Cyp27* gene is expressed in McArdle RH7777 cells, two sets of primers specific for the rat *Cyp27* mRNA were synthesized and used in RT-PCR reactions. As shown in Figure A-4, both the 5' and 3' regions of the *Cyp27* mRNA were detectable in RNA extracted from rat liver but not from McArdle RH7777 cells, whereas the *G3PDH* mRNA was detectable in RNA from both rat liver and McArdle RH7777 cells. These results suggest that the inability of parental McArdle RH7777 cells to convert cholesterol into bile acids via the alternate pathway is attributable to deficiency or severe impairment in Cyp27 activity.

Figure A-4. Detection of the Cyp27 mRNA in rat liver and McArdle RH7777 cell RNA. RNA from rat liver and McArdle RH7777 cells was analyzed by RT-PCR. An ethidium bromide stained agarose electrophoresis gel containing the *in vitro* amplified cDNA corresponding to the 5' and 3' regions of Cyp27 cDNA is shown (top right). The G3PDH cDNA amplified in control reactions is also indicated (bottom right). DNA size markers (M) are in 100 bp increments. The position of the 600 bp size marker is indicated on the left.

Discussion

It has long been considered that Cyp7a1 is the rate-limiting step in the major bile acid biosynthetic pathway (Myant and Mitropoulos, 1977; Russell and Setchell, 1992; Vlahcevic *et al.*, 1999). The results from this chapter have demonstrated that the expression of Cyp7a1 is sufficient to restore limited bile acid biosynthesis in McArdle RH7777 rat hepatoma cells. This finding demonstrates that the complement of enzymatic activities necessary to convert cholesterol into bile acids, including their conjugation with taurine and glycine, have remained in this cell line. Figure A-5A represents a model of the major pathway showing only the two bile acid synthetic enzymes, Cyp7a1 and Cyp27, whose expression has been considered in this study. Also shown are models for what this study has revealed about the major bile acid pathways in both the parental and recombinant McArdle RH7777 cell

lines. For the parental cell line, as predicted, no bile acids are produced. The very first step, Cyp7a1, is not expressed and therefore radiolabeled sterols, which could represent metabolic intermediate products, are also not detected. The model in Figure A-5B demonstrates that since Cyp27 is also not expressed in the parental cell line, bile acid production is also unavailable by these means. The addition of Cyp7a1 to McArdle RH7777 has no effect on the alternate pathway since Cyp7a1 does not play a role in the alternative bile acid synthetic pathway. Rather hydroxylation at the 7 position of the steroid in this pathway is accomplished by an oxysterol 7α-hydroxylase (Cyp7b1) whose substrate specificity would exclude cholesterol catalysis. However, one synthetic enzyme that is shared by both pathways is the microsomal Cyp27. Cyp27 is the initial step in the side chain cleavage in the major pathway and it is also thought to permit the entry of cholesterol into the alternate pathway by converting it into 27-hydroxycholesterol (Wachtel *et al.*, 1968; Wikvall, 1984; Pikuleva *et al.*, 1998). The reduced specificity of oxysterol hydroxylases, other than Cyp27, toward cholesterol suggests a mechanism that ensures a tight control over the conversion of cholesterol into bile acids via the alternate pathway. The mRNA expression of Cyp27 was not detectable in the rat hepatoma cell line used for this study (Figure A-4) likely due to an overall extinction of cytochrome P450 expression. Nonetheless, for parental McArdle RH7777 cells, the lack of Cyp27 expression does not impact the major pathway due to the absence of the first step well upstream of Cyp27.

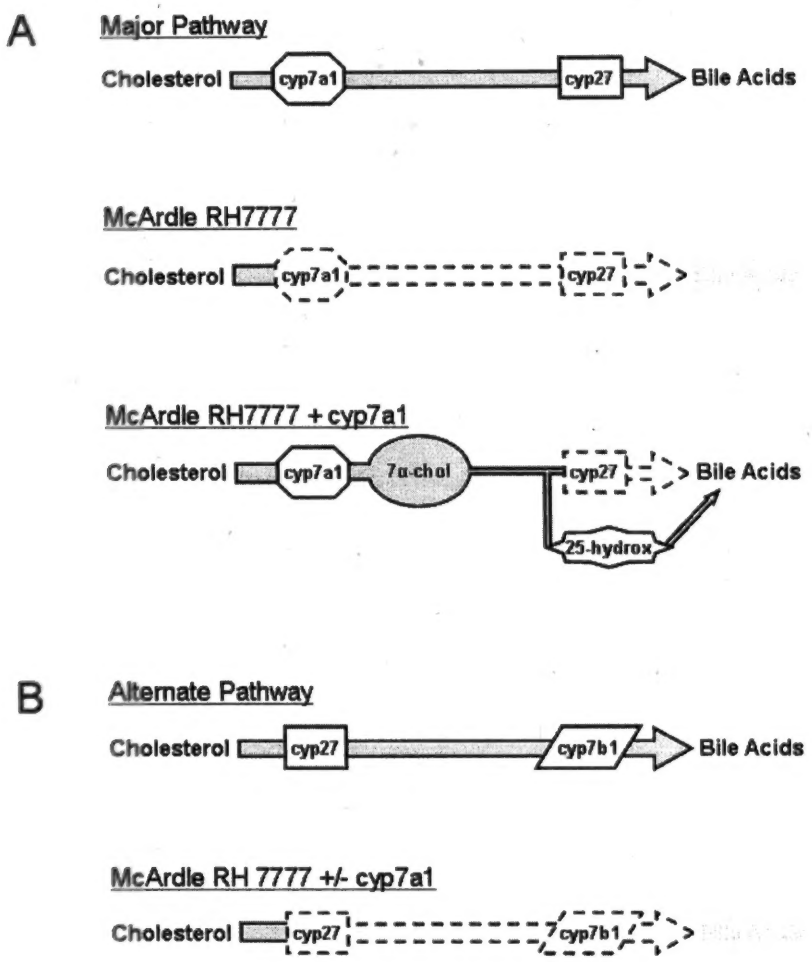

Figure A-5. Models for the major and alternate biosynthetic bile acid pathways in the McArdle RH7777 cell lines. (A) The major pathway of cholesterol conversion to bile acids showing only two of the synthetic enzymes, Cyp7a1 and Cyp27. Underneath are models for both the parental and recombinant cell lines. Note the 7α-hydroxycholesterol (7α-chol) metabolic intermediate build up and the sterol 25-hydroxylase (25-hydrox) which is known to be able to substitute for Cyp27 at this point of the major pathway. (B) The alternate pathway of cholesterol conversion to bile acids showing only two of the synthetic enzymes, Cyp27 and Cyp7b1. Underneath is a model of the alternate pathway which is similar for both cell lines in this study.

As illustrated in Figure A-5A, the accumulation of 7α-hydroxycholesterol in the culture medium of Cyp7a1-expressing cells suggests that the step immediately following the reaction catalyzed by Cyp7a1 is impaired in this cell line. This reaction, which yields 7α-hydroxy-4-cholesten-3-one, is thought to be catalyzed by 3β-hydroxy-Δ^5C_{27}-steroid oxidoreductase (Russell and Setchell, 1992). It is possible that the impairment is due to decreased enzyme activity inherent to McArdle RH7777 cells. Alternatively, it might be the result of active inhibition of enzyme activity by the products of the bile acid biosynthetic pathway. Many synthetic enzymes in both the alternative and major pathways have been found to be negatively regulated, in activities and mRNA expression, by the end products of these pathways. For example, it was demonstrated that sterol 12α-hydroxylase activity is inhibited by bile acids (Vlahcevic *et al.*, 2000). A more recent example shows that Cyp7b1 activity and mRNA levels are reduced by bile acids despite the fact that Cyp7b1 does not seem to be the rate limiting step in the alternative pathway (Pandak *et al.*, 1996). In the recombinant McArdle RH7777 cells, Cyp7a1 activity remains elevated because the synthetic gene used to express Cyp7a1 is not responsive to inhibition by bile acids. This is an unusual situation since part of the reason that the bile acid biosynthetic pathways are difficult to study is because of the rapid conversion of intermediate products that result in low and usually undetectable levels of these compounds in liver tissue. Nevertheless, since bile acids are produced, despite the build up of 7α-hydroxycholesterol, a complement of enzymes that can complete the rest of the major pathway must still be expressed.

Cyp27 is important in the initiation of cholesterol side chain cleavage in the major pathway (Russell and Setchell, 1992). For this reason, the ability

of Cyp7a1-expressing cells to properly synthesize C_{24} bile acid species is surprising (Figure A-3). The conditioned medium of the recombinant cells did not contain significant amounts of oxysterol derivatives of radiolabeled cholesterol and therefore an impairment of side chain cleavage, similar to HepG2 cells. There is evidence to suggest that side-chain cleavage of cholesterol during bile acid synthesis is not strictly dependent upon hydroxylation of C_{27} carbon. The fact that Cyp27-deficient mice are capable of producing C_{24} bile acids (Rosen *et al.,* 1998) demonstrates that Cyp27 is not absolutely required for the removal of the cholesterol side chain during the formation of bile acids. Human and rat liver homogenates contain a microsomal sterol 25-hydroxylase, which is not a cytochrome P450 enzyme, that enables the conversion of 5β-cholestane-3α,7α,12α-triol to 5β-cholestane-3α,7α,12α,25-tetrol (Cronholm and Johansson, 1970; Bjorkhem *et al.,* 1975; Shefer *et al.,* 1976; Furster and Wikvall, 1999), and the latter compound has been shown to be metabolized into cholic acid (Shefer *et al.,* 1976). The microsomal sterol 26-hydroxylase and the sterol 24-hydroxylase may also facilitate side-chain removal. Despite the putative presence of these enzymes, the absence of Cyp27 likely reduces the overall efficiency of side chain cleavage. It is probable that if substrate were not accumulated upstream in the pathway in the form of 7α-hydroxycholesterol, the side chain cleavage mechanism still present in this cell line would be overwhelmed and C_{27} sterol bile acids would be detected in the conditioned medium. The stable expression of functional Cyp27, in addition to Cyp7a1, may improve the side chain cleavage efficiency and the overall yield in bile acid production from the major pathway. Moreover, the accumulation of 7α-hydroxycholesterol may be reduced.

Since the Cyp27 gene expression is impaired in McArdle RH7777 cells, the expression of Cyp7a1 in this cell line largely reconstitutes the major bile acid biosynthetic pathway. Thus, this cell line may be a useful liver cell model for cerebrotendinous xanthomatosis, a condition that is characterized

by Cyp27 deficiency (Russell and Setchell, 1992). Stable expression of only Cyp27 in McArdle RH7777 cells should also permit the selective reconstitution of the alternate pathway. What is unexpected from the results of this study is that CDCA is made in a rat hepatoma cell line. Previous studies from rat hepatocytes indicate that just before extinction of bile acid production muricholic acids and CA were the main products and that CDCA was only produced in small amounts (Hylemon *et al.*, 1985; Princen *et al.*, 1989). Muricholic acid standards were not used in this study since the major bands had been identified using readily available, more inexpensive standards. However, it should be noted that the band identified as TCA does appear rather large in Figure A-3 and could have a more polar taurine conjugated muricholic acid resolving just below it. Additionally, unlike rat hepatocytes, the production of bile acids in this cell line does not decline with culture time. More recently, the problem of bile acid synthetic enzyme extinction of expression was attempted to be alleviated by creating a hybrid cell line from rat hepatoma cells and human fibroblasts (Monte *et al.*, 2001). While this cell line was effective in producing a human compliment of bile acids, this cell line also displays a lack of similarity to normal physiological hepatocytes demonstrated by the inability to conjugate the bile acids produced. McArdle RH7777 cells expressing Cyp7a1 described here could be useful not only in studies addressing bile acid synthesis itself but also in studies assessing the interrelation of bile acid and cholesterol metabolism.

The results demonstrate that McArdle RH7777 cells made to express recombinant Cyp7a1 constitutively are able to synthesize and secrete bile acids into the culture medium. If the loss of Cyp7a1 could be restored in this cell line, it may be a more effective model for bile acid production studies than HepG2 cells or primary hepatocytes. In addition, since bile acid production is species specific, it may provide a more relevant model for rodent bile acid biosynthesis.

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